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OBSERVATIONS ON A NEW ANTIHISTAMINIC COMPOUND POSSESSING UNUSUAL DURATION OF ACTION IN THE GUINEA PIG*

S. WALTER LANDAU AND LESLIE N. GAY

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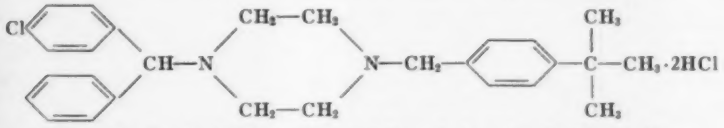
Received for publication March 28, 1955

Efforts directed toward improving the results of antihistamine therapy have brought about the synthesis of many new compounds but have failed to produce a noticeable advance in clinical results. A somewhat unexpected development was the increase in the length of time over which some of these newer compounds exerted their action. This increase was observed in the case of promethazine hydrochloride (Phenergan) (1, 2) and chlorcyclicine (Di-Paralene, Perazil) (3, 4). Recently, in an investigation of a series of 42 methyl piperazine derivatives, one was shown to be effective, in an oral dose of 12 mg., against histamine bronchospasm of guinea pigs for 7–11 days (5). No further experimental data were given.

The following report deals with a compound which, through a single dose, protected guinea pigs against effects of histamine and prevented anaphylactic reactions for from one to three weeks. The compound, 1-parachlorobenzhydryl-4-p-tertiary-butylbenzyl piperazine dihydrochloride (see structural formula) was given the code number AH-2526†. AH-2526 is a white crystalline powder, insoluble in water, but soluble to a limited degree in propylene glycol.



1-parachlorobenzhydryl-4-p-tertiary-butylbenzyl piperazine dihydrochloride

A. ANTIHISTAMINIC ACTIVITY

1. Tests in Vitro—Dale Tests

Procedure: The insolubility of AH-2526 in water prevented the usual procedure of testing the effectiveness of a drug in preventing or relaxing the histamine-induced spasm of the guinea pig gut by adding small amounts to the Dale bath. Instead, a 2 per cent solution of the compound in propylene glycol was administered to the guinea pig intraperitoneally, or the powder was mixed with baby food carrots and given orally. Guinea pigs weighing 250–400 grams were used in all the experiments. The animals were sacrificed from one to five days

* These investigations were made possible by a grant from the Abbott Laboratories.

† The compound was supplied by the Abbott Laboratories through the kindness of Dr. George H. Berryman.

later and a strip of ileum from each one was suspended in Tyrode solution containing atropin sulfate in a final concentration of one to ten million, and aerated with 95 per cent O_2 and 5 per cent CO_2. Two series of experiments were performed. Each of the two procedures was repeated on four to six different days with several pairs of animals. In the first series an intestinal strip of an untreated and one of a treated animal were suspended simultaneously in a bath holding 100 cc. Tyrode solution. In a second series intestinal strips of an untreated animal were tested with histamine until the maximal effect of a certain dose of histamine was ascertained. This was done in a small tissue bath holding 3 cc. Tyrode solution. Similarly, strips of a treated guinea pig were suspended and that dose of histamine which produced a contraction of the same height as had been produced in those of the untreated animal was determined. This procedure was then repeated on a second and frequently on a third pair of guinea pigs, one treated and one untreated. In one group of experiments, guinea pigs were injected intraperitoneally with 20 mg./kg. of the compound from one to five days before carrying out the Dale tests. When it became apparent that the solubility of the compound in propylene glycol was incomplete and that sometimes unabsorbed particles were found in varying amounts in the peritoneal cavity when the gut was removed, the results were checked in a second group of animals to which five to twelve mg. of AH-2526 per animal was given orally.

Results: There was no difference between the effect of 20 mg./kg. given by injection or 12 mg. of the compound given by mouth one to five days prior to the test. In both cases the effect of a dose of histamine* which produced a strong contraction of the control gut suspended in the same bath was completely suppressed (Fig. 1a, b).

In testing intestinal strips successively in the 3 cc. bath, it was found that the "treated" strip required approximately 500 times as much histamine to produce contractions of the same height as those obtained with the "untreated" strip. Whether the compound was given intraperitoneally or orally, or whether the test was carried out one or five days after the administration of the compound, did not affect the 1–500 ratio. Obviously, the dose of 20 mg./kg. was sufficiently large to make inconsequential any loss due to insufficient solubility, or through incomplete absorption.

In contrast to the suppression of the histamine contraction, the "treated" gut in most instances responded as strongly to acetylcholine as did the normal gut. (The atropine was, of course, omitted in these tests.) Only in a few tests was a slight decrease of response in the gut of a treated animal observed.

2. Tests in Vivo

a. Histamine aerosol inhalation

Procedure: A histamine solution containing 0.5 mg./cc. histamine base was blown under a constant pressure of 5 lbs. (derived from a compressed air tank) into a cylindrical glass chamber occupied by the guinea pig. The shape of the atomizer containing the histamine

* All values of histamine are expressed as histamine base. Histamine acid phosphate or histamine dihydrochloride was used.

a) b)

FIG. 1. Dale test, intestinal strip of guinea pig. *Upper* tracing—untreated guinea pig. *Lower* tracing—guinea pig fed 12 mg. AH-2526. a) One day before test. b) Three days before test. At ↑ 0.5 cc. of 1 gamma/cc. histamine added to 100 cc. Tyrode solution.

solution was similar to that of the commercial vaponefrine atomizer.* Under such conditions, normal guinea pigs of 250–400 grams weight reveal symptoms in the following order: Dyspnea, i.e., accelerated respiration; asthma, i.e., slow labored breathing with drawing in of the flanks; convulsions; weakness; and finally, death. The earliest users of the aerosol inhalation test accurately observed that the guinea pigs inhaling the histamine mist first exhibit an increase then a decrease in frequency of respiration accompanied by gasping movements of the body. (6, 7) We have consistently noticed these two stages of respiratory changes, and for convenience, have used the term "dyspnea" for the first and the term "asthma" for the second. In the majority of the animals, dyspnea commences from 40–70 seconds after the aerosol has started to enter the chamber; occasionally, about two minutes elapse before breathing becomes accelerated. If an animal fails to show definite response within this time, it is unusually resistant to histamine and is unsuitable for testing. The asthmatic state usually follows the period of dyspnea within one minute. Some of the animals cough, some scratch occasionally or for a prolonged time, most lie on their side after several minutes' exposure and remain so until death; others get up again for a short time. Convulsions do not occur in all animals. They may appear early, may be mild and recur a number of times, or they may be violent and continuous. In a series of nine normal guinea pigs exposed to histamine aerosol as described, we found that in spite of usual behavior as to the early symptoms, the time interval until death varies considerably—from 4½ to more than 20 minutes.

It has been reported that repeated exposure of guinea pigs to histamine aerosol does not result in increased tolerance, particularly if not less than seven days elapse between the tests. (8, 9). In our experiments, the guinea pigs showed at times a slight decline in sensitivity when the test was repeated at intervals of two to six days: the number of animals that suffered convulsions decreased somewhat, and a slight increase occurred in the time required for onset of dyspnea. These changes, however, were of such a minor degree that no difficulty arose in comparing the change in behavior of the animals caused by administration of the compound. It can be confirmed here that in any series of tests one or more of the animals may become "conditioned" (10) to the experiment, for as soon as placed in the chamber they become excited and start to breathe heavily, even before the histamine aerosol reaches them. Such animals were excluded from the experiments.

The results of this testing procedure can be evaluated by two methods: either by observing the symptoms which developed within a fixed period of exposure, or by determining the time required for the first definite symptoms to appear. Each method has its advantages and its disadvantages; therefore, a combination of the two was decided upon. In a first series each animal in the pre-drug test was kept in the chamber for five minutes, unless severe convulsions necessitated an earlier removal; in the test following the administration of the compound, all animals were kept in the chamber for ten minutes. In the subsequent series each animal in the pre-drug tests was removed as soon as definite dyspnea was present. This occurred within two minutes. In many instances the symptoms increased after removal, progressing to cough, asthma, and even convulsions. After AH-2526 had been given, each animal was kept in the chamber for ten minutes. This time arrangement increased the severity as well as accuracy of the test, for symptoms, the onset of which might have been delayed because of the effect of AH-2526, could become noticeable in an exposure usually five times as long as that endured before administration of the compound. After AH-2526 had been given, one to five days were allowed to elapse before the animals were retested with

* We wish to express our gratitude to Dr. F. F. Yonkman and Dr. R. Mayer of the Research Department of the Ciba Pharmaceutical Products, Inc. for the loan of their chamber and atomizer.

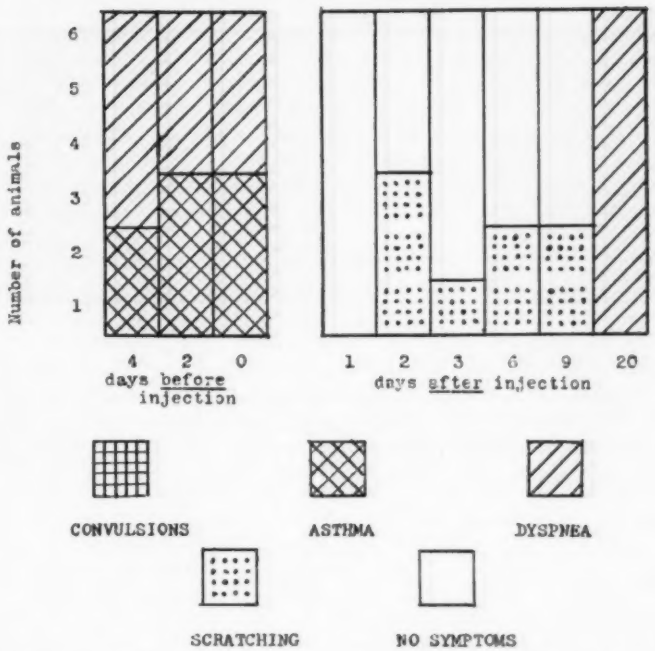


FIG. 2. Response of guinea pigs to histamine aerosol before and after i.p. injection of 20 mg./kg. AH-2526 (2nd series). Animals exposed before injection until first symptoms appeared; after injection, for 10 minutes.

histamine aerosol and exposure was repeated up to thirty-three days after drug administration.

Results: Figures 2–4 illustrate the marked reduction and the very gradual reappearance of the more severe symptoms from histamine aerosol when 20 mg./kg. of AH-2526 was injected prior to histamine exposure. In the six animals whose responses are shown in Figure 2 there was no indication of tolerance developing to histamine in three trial (pre-drug) exposures given in short intervals. However, nine days after administration of AH-2526 the animals were still free from major symptoms and even after 20 days though all developed dyspnea, no "asthma" was noted when exposed in the chamber for 10 minutes. Figure 4 indicates the considerable delay in the development of dyspnea 12 days after the injection (there was no dyspnea five and eight days after the injection), and the very slow return to the original short interval between exposure to histamine and appearance of dyspnea. When 10 or 20 mg. of the compound was fed to the guinea pigs, there was a similar suppression of symptoms for at least one week.

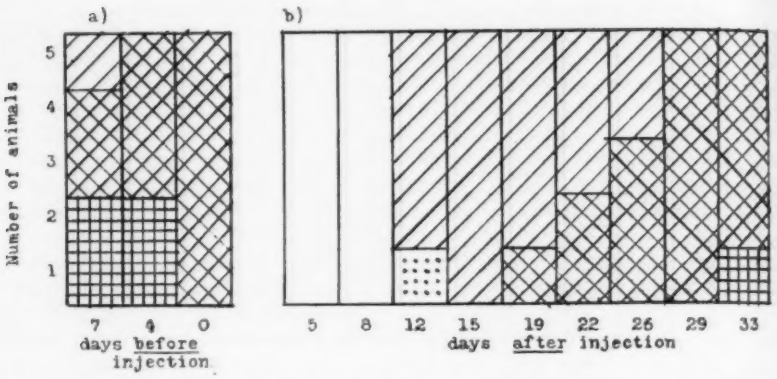


FIG. 3. Response of guinea pigs to histamine aerosol before and after i.p. injection of 20 mg./kg. AH-2526 (3rd series). Animals exposed before injection until first symptoms appeared; after injection, for 10 minutes.

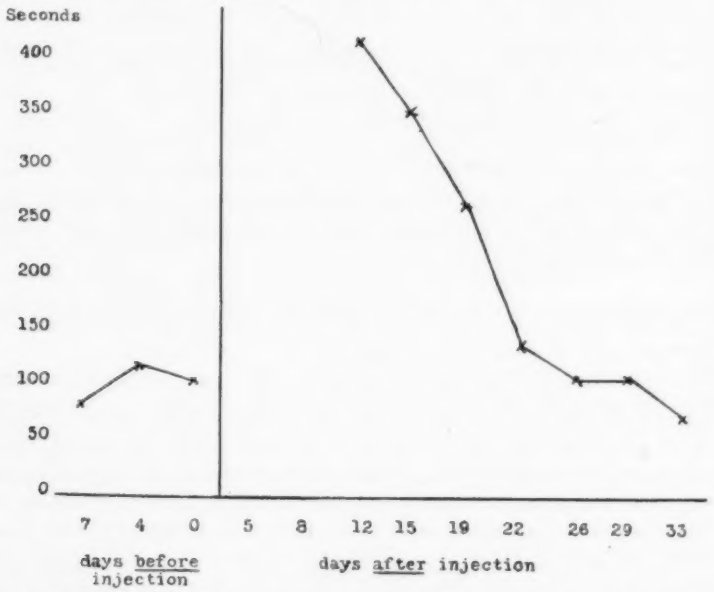


FIG. 4. Time of onset of dyspnea from histamine aerosol before and after 20 mg./kg. AH-2526 i.p. (3rd series). Average values of five guinea pigs.

b. Administration of lethal doses of histamine i.v.

Procedure: According to our experience a dose of 0.5 mg./kg. histamine base injected intravenously will kill usually 100 per cent of normal animals of 250–400 gram weight. This figure was verified in ten animals which died within two minutes after the injection. Four additional guinea pigs died from the same dosage after they had received 0.5 cc. propylene

glycol intraperitoneally, the amount used as vehicle for the injections of AH-2526 (Table I). In this report, therefore, the term lethal dose refers to 0.5 mg./kg. histamine base given i.v. All injections of histamine were given in the jugular vein, exposed under local anesthesia.

In one large series of experiments, AH-2526 was injected i.p. in varying concentrations, and the i.v. injection of one or more lethal doses of histamine was given on the next day or on an increasing number of days later. In a second series of experiments, the compound was administered orally one or more days prior to the injection of histamine. Autopsies were done on all the animals that died.

Results: Tables I and II summarize the results which were obtained when AH-2526 was injected or fed. Injections of 1 mg./kg. of the compound com-

TABLE I

Effect in Guinea Pigs of Varying Doses of AH-2526 (Given i.p.) on One or Multiple Lethal Doses of Histamine (Given i.v.)

Number of Days Following Injection of AH-2526	Amount of AH-2526	Number of Lethal Doses of Histamine	Survived	Died
	mg./kg.			
	None (controls)	1	0	10
1	Propylen glycol (controls)	1	0	4
1	1	1	5	0
	1	2	2	1
	2	2	2	0
	2	4	4	2
	20	1	8	0
2	20	1	7	0
3	5	10	5	3*
7	20	1	4	0
	20	2	4	0
	20	4	2	0
	20	10	7	0
	20	20	6	0
8	1	1	6	4*
	1	2	0	1
	2	2	6	0
	5	5	4	1
11	20	20	2	0
	20	40	8	0
14	20	20	6	0
21	20	20	7	2†

* One delayed death.

† Two delayed deaths.

TABLE II

Effect in Guinea Pigs of 5 mg. AH-2526 (Given Orally) on One or Multiple Lethal Doses of Histamine (Given Intravenously)

Number of Days Following Feeding of AH-2526	Number of Lethal Doses of Histamine							
	One		Two		Five		Ten	
	Survived	Died	Survived	Died	Survived	Died	Survived	Died
1	2	0	2	0	2	0		
4					6	0		
5			2	0	10	0		
8	8	0					9	2*
12					8	0		
14	9	1					0	5*
19	1	4*						

* One delayed death.

pletely protected against one lethal dose of histamine given one day later, but only partially against two lethal doses. The same relationship held true for multiples; e.g. 2 mg./kg. AH-2526 protected completely against two lethal doses but only partially against four lethal doses of histamine, and so forth. This degree of activity would rank this compound as a weak antihistaminic compared with others in their protective action in the guinea pig. The outstanding feature, however, was the duration of its protective effect. Fourteen days after injection of 20 mg./kg. AH-2526 into six guinea pigs there was no death from 20 lethal doses of histamine, and even 21 days after injection of 20 mg./kg. AH-2526 seven out of nine guinea pigs survived (Table I). A single oral dose of 5 mg. of the compound was also effective for an unexpected length of time: there was complete protection against one lethal dose of histamine for 14 days, and against five lethal doses for 12 days and protection was almost complete against ten lethal doses for eight days (Table II).

B. ANTIANAPHYLACTIC ACTIVITY

1. Tests in Vitro—Dale Tests

Procedure: Guinea pigs were sensitized both actively and passively. In the experiments employing actively sensitized animals, two doses of 0.2 cc. hog serum were given i.p., four to five days apart. Three and one half weeks later, 20 mg./kg. AH-2526 in propylene glycol was injected i.p. The following day the guinea pig gut was tested with a hog serum dilution, 1:10. In the experiments utilizing passive sensitization, 2 cc. antiovalbumin rabbit serum was injected i.p. This serum had an antibody titer (antigen dilution test) of 1:80,000. It was derived from rabbits which had been injected intramuscularly in ten different sites simultaneously with 0.4 cc. (per site) of an emulsion of fresh egg white in Freund's adjuvant (Formula: egg white, 8 cc.; aquaphor, 4 cc.; mineral oil, 16 cc.; killed tubercle bacilli, 20 mg.)* and had been bled three weeks later. One day or two days after passive sensitization,

* We are grateful to Dr. M. Mayer of the Johns Hopkins School of Hygiene for supplying the killed tubercle bacilli.

AH-2526 was administered intraperitoneally or orally, and one day or several days later the gut was tested in the bath with a solution containing 0.1 mg./cc. crystalline ovalbumin.

Similarly to the histamine tests, several pairs of guinea pigs, one treated and one untreated, were used on each day, and tests were carried out on each of seven days. Because tests with antigen cannot be repeated on the same strip, and different strips of the same animal gut frequently have a different and an unpredictable degree of sensitivity to the same dose of antigen, there are difficulties in comparing the response of strips from treated with those from untreated animals. Other investigators (11) have observed that in a given strip from a sensitized guinea pig, the sensitivity to histamine does not parallel the sensitivity to the antigen. Therefore sensitivity to histamine cannot be used as a standard for comparison. We have repeatedly encountered segments of ileum that gave poor response to histamine but showed excellent contractions when tested with the antigen. This phenomenon is of considerable theoretical interest. The test dose of the antigen was so selected that no maximal contraction occurred in any of the intestinal strips, as otherwise a distorted picture of the relative sensitivity of the two guts would have been produced. Since strips originally cut in equal lengths frequently relax in the bath to a different extent, this factor received close attention and every effort was made to compare strips of the same final length. In all tests with antigen the 100 cc. bath was used, and a strip from an untreated and one from a treated guinea pig were suspended simultaneously.

Results: There was no essential difference between the results observed in guinea pigs actively sensitized and in those passively sensitized. Likewise, there was no essential difference between results obtained by intraperitoneal administration of 20 mg./kg. and those obtained by feeding 12 mg. per animal. In all instances, when the antigen was added to the bath, the gut from the treated animal either contracted considerably less than the "untreated" gut or failed completely to contract (Fig. 5). This was true whether the tests were carried out the day after the compound had been given or 3–4 days later. In those cases in which the "treated" gut did react, the contraction most frequently seen reached 20 per cent of that of the "untreated" gut, but in a few instances the contraction was as high as 50 per cent. Taking into consideration the difficulties involved in making such comparisons as mentioned above, we can conservatively state that i. p. injection of 20 mg./kg. of AH-2526 or oral administration of 12 mg. per animal reduces the antigen response in the gut of an actively or a passively sensitized guinea pig by 50–100 per cent, for at least four days.

2. Tests in Vivo

a. Antigen aerosol inhalation

Procedure: Thirteen guinea pigs were sensitized actively with two injections of 0.2 cc. hog serum given intraperitoneally four days apart. Four weeks later the animals were divided at random into two groups. Seven animals were given 20 mg./kg. AH-2526 i.p., six animals served as controls. Two days, ten days, and thirteen days later all animals were exposed to an aerosol of hog serum diluted 1:2 for five minutes. Passive sensitization was carried out in another group of 13 guinea pigs by injecting i.p. 3 cc. of antiovalbumin rabbit serum. For pre-drug testing, six animals were exposed in the aerosol chamber to a vaporized solution of

FIG. 5. Dale test, intestinal strip of guinea pig passively sensitized to ovalbumin. *Upper* tracing—untreated guinea pig. *Lower* tracing— guinea pig given 12 mg. AH-2526 orally 3 days before test. At ↑ 0.5 cc. of 0.1 mg./cc. ovalbumin added to 100 cc. Tyrode solution.

10 mg./cc. crystalline ovalbumin. On the following day each was fed 12 mg. of the compound. Three days after the feeding, and again two days later, the aerosol test was repeated in these six animals, and the seven remaining animals were also exposed to the antigen aerosol as controls. In the pre-drug testing and in all tests of the controls, each animal was removed as

soon as symptoms appeared. In the two tests following the feeding, the treated group was kept in the chamber for 10 minutes.

Results: In the actively sensitized animals injection of 20 mg./kg. AH-2526 caused marked suppression of symptoms for at least 13 days. There was not only lack of asthma or convulsions in a 5 minutes exposure, but also marked delay in the appearance of dyspnea compared with the controls. Most of the controls developed asthma, some also convulsions within 2 or 3 minutes after starting the test.

Figure 6 illustrates the effect of feeding the compound to the passively sensitized guinea pigs. Before feeding they all had severe reactions to the aerosol even when removed from the chamber at once when the first signs of dyspnea appeared. Three and five days after the feeding, there was complete freedom from symptoms or only a minor reaction during an exposure to the antigen for 10 minutes. When the controls were tested the second time, the severity of reactions was somewhat diminished, probably because in repeated tests some desensitization has occurred and reformation of antibodies has taken place only partially, as suggested by Herxheimer (10). However, this reduction in the degree of symptoms is insignificant compared with that seen in the same animal after feeding the compound.

b. Induction of lethal anaphylactic shock i.v.

Procedure: A group of guinea pigs was actively sensitized with two injections of 0.2 cc. hog serum three to five days apart. From three to four weeks later, they were challenged

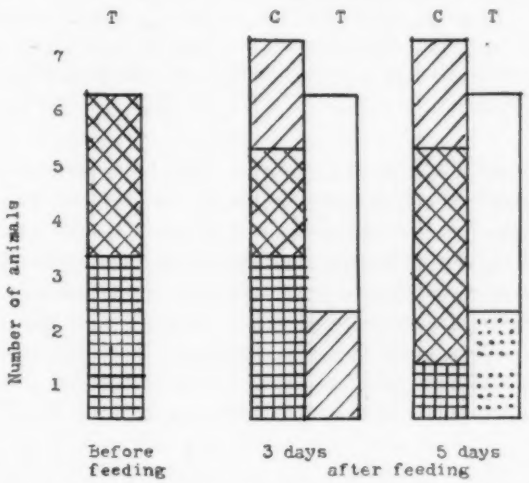


FIG. 6. Response of guinea pigs to antigen aerosol after oral administration of 12 mg. AH-2526—passive sensitization to ovalbumin. C—Control group. T—Treatment group. The treatment group before feeding and the control group exposed until first symptoms appeared; the treatment group after feeding exposed for 10 minutes.

TABLE III

Effect of Compound AH-2526 on Anaphylactic Shock in Guinea Pigs

Number of Days Following Administration of AH- 2526	Amount and Route of Administration of AH-2526	Immediate Death	Delayed Death	Survival	Percent of Survival
		A. *Active Anaphylaxis*			
	None (controls)	8	0	0	0
1 or 2	20 mg./kg. i.p.	0	3	2	40
		B. *Passive Anaphylaxis*			
	None (controls)	22	0	0	0
1 or 2	20 mg./kg. i.p.	0	6	4	40
	40 mg./kg. i.p.	0	1	7	87
	12 mg. orally	0	1	6	86
6	40 mg./kg. i.p.	0	1	3	75
	12 mg. orally	0	2	8	80
9	40 mg./kg. i.p.	1	0	6	85
12	12 mg. orally	0	0	8	100

with 0.5 cc. hog serum injected into the jugular vein. Passive sensitization was carried out in other groups of guinea pigs by injecting 2 cc. of antiovalbumin rabbit serum i.p. This serum, obtained from rabbits sensitized as described above, had an antibody titer of 1:80,000 or higher. From one to three days after the sensitizing injection, these animals were challenged with an intravenous injection of 2 mg. crystalline ovalbumin in 0.4 cc. distilled water. Table III shows the mode of administration of AH-2526.

When protection from 20 mg./kg. AH-2526 was found unsatisfactory, the dose was increased to 40 mg./kg. given intraperitoneally, or 12 mg. per animal was fed. The original time interval of one to two days between administration of the drug and intravenous challenge was extended to six, nine and twelve days respectively. Autopsies were done in all animals that died.

Results: As seen in Table III, 100 per cent of the 30 controls died from anaphylactic shock; death occurred within two or three minutes. After injection of 20 mg./kg. AH-2526, 40 per cent of the experimental animals survived. Injection of 40 mg./kg. or feeding of 12 mg. of the compound achieved 75–100 per cent survival up to twelve days later. Of the 58 animals treated with AH-2526, only one died in acute anaphylactic shock with the typical picture of huge inflated lungs; fourteen animals died after 20–40 minutes or more, and the autopsy showed the small lungs with hemorrhagic surface and frequently the dark congested guts common in delayed anaphylactic death.

c. Arthus reactions

Procedure: The method of Benacerraf and Kabat (11), inducing the Arthus phenomenon passively in the guinea pig, was used with minor modifications. Twelve guinea pigs were in-

jected i.p. with 2 cc. antiovalbumin rabbit serum containing 0.49 mg. antibody nitrogen.* Six of these animals had received 40 mg./kg. AH-2526 i.p. three days before. One day after injection of the rabbit serum, the 12 animals were injected intracutaneously with 1 mg. crystalline ovalbumin in 0.2 cc. salt solution.

A second group of five guinea pigs received i.p. 2 cc. of antiovalbumin rabbit serum containing 0.49 mg. antibody nitrogen, and two days later 1 mg. crystalline ovalbumin in 0.2 cc. was injected intracutaneously. Four days after injection of ovalbumin 40 mg./kg. AH-2526 was injected i.p. into the five animals. Six days after the administration of the compound one mg. ovalbumin was again injected intracutaneously. Ten days after the administration of the compound the animals were challenged with 2 mg. crystalline ovalbumin intravenously.

Results: In the first group of 12 animals, a positive Arthus reaction was present four hours after the injection of the ovalbumin. The degree of the reaction was equal in the animals that had received AH-2526 four days earlier and in the controls. The 12 guinea pigs had edema at the site of injection approximately 25 x 35 mm. in size. Of the six control animals, three had petechial hemorrhage; of the six treated animals, four had petechial hemorrhage at the sites of injection.

In the second group of five animals, the Arthus reactions elicited six days after administration of 40 mg./kg. AH-2526 were equal to those which occurred preceding the use of the compound. However, when challenged i.v. with ovalbumin ten days after injection of the compound, only mild anaphylactic symptoms developed, and all animals survived.

DISCUSSION

If we recall that one of the early antihistaminic drugs, pyranisamine (Neo-Antergan) in as small a dose as 0.02 mg./kg., when given subcutaneously half an hour before the histamine injection, achieved a 100 per cent protection against one intravenous lethal dose of histamine (2), the antihistaminic activity of the new compound would appear far from impressive. Experience has taught, however, that clinical activity does not follow the dosage scale which prevails in guinea pigs against the effects of histamine. The protection which AH-2526 gave to guinea pigs against fatal anaphylactic shock was of a higher order, but still required eight times the dose necessary with pyranisamine. Recognizing that this comparison in amount of injected dose is only approximately correct, for AH-2526 could not be administered in very accurate doses because of its incomplete solubility in the vehicle propylene glycol, we may generally conclude from our experiments that relatively high doses of this new compound have to be used in guinea pigs to achieve inhibition of histamine effects and of the effects of antigen-antibody reactions.

* The quantitative precipitin test was carried out in the laboratories of the Johns Hopkins University School of Hygiene through the kindness of Dr. M. Mayer.

No difficulty was encountered in using a high dosage level, for 40 mg./kg. administered intraperitoneally or 12 mg. given orally caused no noticeable side effects in the animals. According to written communication received from the Abbott Laboratories, the acute toxicity of the drug (given orally to mice) was only about one-fifth that of tripelenamine (Pyribenzamine) or of chlorcyclizine (Di-Paralene, Perazil).

The outstanding and unusual property of the compound AH-2526 proved to be the duration of the effect of a single dose. The suppression of the smooth muscle contraction due to histamine for at least five days and the resistance to aerosoled histamine (complete for eight or nine days and partial for about three weeks) were matched by the protection given against 20 i.v. lethal doses of histamine, a protection complete for at least two weeks and almost complete at the end of three weeks. All these effects came from a single dose of 20 mg./kg. of the compound, given i.p. Protection against death from passive anaphylaxis was afforded by 40 mg./kg. of the compound for at least nine days. At first, a very slow absorption of the poorly soluble material from the peritoneal cavity was considered as an explanation. This idea of a depot action had to be dismissed when it became apparent that oral administration of a single dose also had a very prolonged effect, though in somewhat lesser degree. The fact that guinea pigs after ingestion of 5 mg. of AH-2526 were completely resistant against five lethal doses of histamine not only for four to five days but even for 12 days, is certainly astonishing. Likewise, after one oral dose of 12 mg. of AH-2526, there was no death from anaphylactic shock in passively sensitized guinea pigs 12 days later (100 per cent of the controls died).

Roth and his colleagues (4) considered the possibility that their drug chlorcyclicine might inhibit to an unknown degree the sensitization normally achieved by the sensitizing injection of antiserum. Such an eventuality, of course, would not apply to our experiments on animals actively sensitized, for AH-2526 was given three or four weeks after the sensitizing injections. Using passively sensitized animals, in the Dale tests, we administered the drug one or two days after the injection of the antiserum at a time when it would be expected that sensitization had taken place. That sensitization achieved was proved in the antigen aerosol tests, for exposure of the treatment group before administering the compound produced the same degree of reactions as that seen in the control group. It would, therefore, seem far-fetched to assume that the protection achieved in those guinea pigs to which AH-2526 had been given *before* the sensitizing dose of antiserum was due to inhibition of sensitization rather than to inhibition of effects from the antigen-antibody reaction. The results of the experiments with the Arthus reaction proved further that sensitization had taken place without interference from AH-2526.

Dreisbach (13) found that in rabbits diphenhydramine (Benadryl) and tripelenamine (Pyribenzamine) did not antagonize the Arthus reactions pro-

duced by horse serum or penicillin. This author, therefore, doubted whether histamine is liberated in this kind of reaction. Recently, it was shown (14) that even very large injections of histamine into rabbit skin do not produce Arthus type reactions. Our results confirm that an effective antihistaminic drug does not inhibit skin reactions of the Arthus type, and thus indicate that histamine does not play a major role in their origin.

To explain the extremely long duration of activity of this new compound is difficult. One would assume that its radicals remain anchored tenaciously to the effector cells with which liberated or introduced histamine would normally combine. Baltzly and coworkers (15) speculated "that the prolonged action of the para-halogen derivatives might be due to inhibition of metabolic oxidation" or that these derivatives might "function by being absorbed on (and blocking the action of) some enzyme whose matrix admits the presence of para-substituents only".

Results of clinical tests with the compound will be reported elsewhere. Its effect in allergic diseases was not very satisfactory because much higher doses than could be tolerated would have been necessary. If its side effects could be eliminated, a drug of such prolonged activity might be useful in preventing such conditions as serum sickness or delayed penicillin reactions. From a theoretical viewpoint the remarkable duration of action of this compound seems to deserve further studies.

SUMMARY

A single dose of a new antihistaminic compound, AH-2526, administered parenterally or orally to guinea pigs produced the following results:

1. Inhibition to a large degree of the smooth muscle reaction to histamine in the Dale bath for at least five days.
2. Inhibition of all or of the major effects of histamine aerosol for four to nine days and decrease in response to histamine aerosol for three weeks or more.
3. Protection against death from i.v. histamine injection for two to three weeks.
4. Reduction in response of the gut of a sensitized animal to the homologous antigen by 50–100 per cent for at least four days.
5. Diminution in response of sensitized animals to an aerosoled antigen for five to ten days.
6. Protection against death from anaphylactic shock for at least 12 days.
7. Failure to inhibit the passively induced Arthus reaction.

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REFERENCES

1. Landau, S. W. and Gay, L. N.: Comparative studies of antihistamine substances I. Introduction and Dale experiments. Bull. Johns Hopkins Hosp., 1948, **83:** 330.

2. LANDAU, S. W., MARRIOTT, H. J. L. AND GAY, L. N.: Comparative studies of antihistamine substances II. Activity in vivo against histamine intoxication and anaphylactic shock of guinea pigs. Bull. Johns Hopkins Hosp., 1948, **83:** 347.
3. CASTILLO, J. C., DEBEER, E. J. AND JAROS, S. H.: A pharmacological study of N-Methyl-N′-(4-Chlorobenzhydryl) Piperazine Dihydrochloride, a new antihistaminic. J. Pharm., 1949, **96:** 389.
4. ROTH, L. V., RICHARDS, R. K. AND SHEPPERD, I. M.: Pharmacological studies of a new antihistaminic compound, N-(p-Chlorobenzhydryl)-N-Methylpiperazine Hydrochloride. Arch. internat. pharmacod. et de thérap., 1949, **80:** 378.
5. MORREN, H., TROLIN, S., DENAYER, R., GRIVSKY AND MARICQ, J.: Nouvelles substances antihistaminiques à action prolongée; Les 1–4 Bis (Aralcoyl) Piperazines. Bull. Soc. Chim. Belg., 1951, **60:** 282.
6. KALLOS, P. AND PAGEL, W.: Experimentelle Untersuchungen ueber asthma bronchiale. Acta. Med. Scand., 1937, **91:** 292.
7. SCHAUMAN, O.: Ueber eine neue Klasse von Verbindugen mit spasmolytischer und zentral analgetischer Wirksamkeit unter besonderet Beruecksichtigung des 1-Methyl-4-Phenyl-Piperidine-4-Carbonsaure-Ethylesters (Dolantin). Arch. Fuer Exp. Path. und Pharm., 1940, **196:** 109.
8. MAYER, R. L.: Antihistaminic substances with special reference to Pyribenzamine. J. Allergy, 1946, **17:** 153.
9. FRIEBEL, H.: Histamin und anaphylaktischer Schock. Zeitschr. f. Aerosolforschung und-therapie. 1. Beiheft, 1953.
10. HERXHEIMER, H.: Repeatable "microshocks" of constant strength in guinea pig anaphylaxis. J. Physiol., 1952, **117:** 25.
11. KEMPNER, A. H. AND FEINBERG, S. M.: Dissimilar sensitivity to antigen of segments from the isolated intestinal strip. J. Allergy, 1948, **19:** 247.
12. BENACERRAF, M. AND KABAT, E. A.: A quantitative study of the Arthus phenomenon induced passively in the guinea pig. J. Immunol., 1950, **64:** 1.
13. DREISBACH, R. H.: Failure of Benadryl and Pyribenzamine in experimental skin sensitization to Penicillin and horse serum. J. Allergy, 1947, **18:** 397.
14. CROXATTO, O. C.: The failure of histamine to reproduce the Arthus phenomenon. Bull. Johns Hopkins Hosp., 1952, **90:** 349.
15. BALTZLY, R., DUBREUIL, S., IDE, W. S. AND LORZ, E.: Unsymmetrically disubstituted Piperazines. III. N-Methyl-N-Benzhydrylpiperazines as histamine antagonists. J. Organic Chem., 1949, **14:** 775.

THE SYNDROME OF GONADAL AGENESIS (OVARIAN AGENESIS) AND MALE CHROMOSOMAL PATTERN IN GIRLS AND WOMEN: PSYCHOLOGIC STUDIES[1, 2]

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INTRODUCTION

The syndrome named after Turner in 1938 was redefined by Albright (1) in 1942 as a syndrome of ovarian insufficiency and has subsequently been referred to as ovarian agenesis also. During the past 3½ years, 19 patients with a diagnosis of ovarian agenesis have come under our observation for psychologic study. The pertinence of these studies for psychiatric and psychologic theory was dramatically enhanced during the past year when it was discovered that many of the patients had male chromosomes[3] and were, to be precise, cases of gonadal agenesis (or dysgenesis) and not ovarian agenesis. A report on the chromosomal studies will be published separately by Grumbach and others (3).[4]

[1] The research on which this study is based is supported by a research grant from the Josiah Macy, Jr. Foundation and is under the aegis of John C. Whitehorn, Professor of Psychiatry, The Johns Hopkins University, and of Lawson Wilkins, Associate Professor of Pediatrics, Acting Pediatrician-in-Chief, director of pediatric endocrinology at The Johns Hopkins Hospital.

[2] This paper was presented at the Biennial Meeting of The Johns Hopkins Medical and Surgical Association, Baltimore, Md., Feb. 25, 1955.

[3] Chromosomal determinations were done by Dr. M. L. Barr and Dr. K. L. Moore at the University of Western Ontario (2).

[4] The paradoxical incongruity between male chromosomes and female genitalia seems likely to be human counterpart of the phenomena encountered by Jost. In a brilliant piece of research Jost (4, 5) in 1947, demonstrated that embryonic rabbits, castrated in utero prior to the stage of gonadal differentiation, all developed female accessory organs. The effects of castration on the male embryo depended on the date of the operation. Castration on the 24th day of embryological development did not interfere with normal development of male reproductive organs. On the 23rd day, castration inhibited development of the wolffian duct system. But castration before the 21st day was followed not only by complete regression of the wolffian ducts, but also by persistence of the mullerian ducts and their differentiation into oviducts, uterus and vagina. The external genitals took on a feminine configuration. The prostate was arrested in its development, or was completely absent if the operation was performed before the 19th day. Castration of the female, on the other hand did not prevent the acquisition of female accessory reproductive organs. Performed on the 23rd day, castration was not followed by any noticeable effect, but prior to the 21st day it led to a decrease in the size of the mullerian organs, so that their development in the embryos of both sexes castrated so early was essentially equivalent.

Among the anatomical and physiological characteristics of the syndrome of ovarian or gonadal agenesis, the following are particularly relevant in relation to psychologic considerations.

1. Genitals: Untreated the internal and external genitalia are of normal infantile female appearance with the single exception that the gonads are absent, being represented only by a narrow ridge of poorly differentiated non-functioning tissue in each broad ligament.
2. Hormones: There is normal female production of adrenal hormones, increased FSH output at puberty and absence of gonadal estrogens or androgens.
3. Stunted growth: All of the patients thus far diagnosed have been moderately stunted in growth and have ranged in height from 50 to 58 inches.
4. Anomalies: Inconsistently distributed amongst cases, are anomalies such as webbed neck and coarctation of the aorta.

The significance of the syndrome of gonadal agenesis for psychiatric and psychologic theory is that it provides an opportunity to examine the relative importance of chromosomal and hormonal sex as determinants of gender-role. By *gender-role* we mean all those things that a person says or does to reveal himself or herself as boy or man, girl or woman respectively. It includes, but is not restricted to sexuality in the sense of eroticism.

In making a report on the psychologic studies of patients with gonadal agenesis, it has been decided to focus attention on the 11 among the 13 so far biopsied[5] who proved to be chromosomally male. In addition, since no psychologic reports on patients with this syndrome have appeared in the literature, it has been decided that the presentation of a single case in extensive detail will, more cogently than piecemeal documentation from all of the cases, illustrate the salient psychologic findings. The particular case was selected because this patient was the only one who provided any basis for being skeptical of her feminine orientation, owing to a childhood episode of homoeroticism, and yet who, in the ultimate analysis, was like all the other patients in leaving no doubt about her psychological femininity.

PROCEDURE

An evaluation of the human characteristics encompassed by the term gender-role is made difficult by the lack of generally accepted methods and measurements to employ. In this research we have attempted to face up to this methodological handicap in the manner described in an earlier publication (6). Briefly, due consideration was given the following: general mannerisms, deportment and demeanor, play preferences, recreational interests, spontaneous topics of

[5] Since this paper was written the chromosomal sex of 37 patients with gonadal agenesis has been determined. Of these, 31 were chromosomally male and 6 chromosomally female

talk in unprompted conversation and casual comment, content of dreams, daydreams and fantasies, replies to oblique inquiries and projective tests, evidence of erotic practice and finally, the person's own replies to direct inquiry. Interviews with patients and relatives were recorded for later transcription. The frequency of patient follow-up has varied with individual circumstances but usually patients were seen at intervals of 6 months or a year.

CASE ILLUSTRATION

Introductory data: The patient was referred for endocrine study at the age of 11 yr. 5 mo., with the complaint that she was sluggish, doing poor work in school and not growing as fast as she should. The first psychological studies were done at the age of 12 yr. 4 mo., with the subsequent intermittent follow-up over a 3-year period. HLH # A-88683.

At the age of 11 yr. 5 mo. the following data were recorded. Height 3 ft. 11.3 in. Height age 7¼ yr. Bone age 11 yr. No sexual development. Dental development normal for age. She had a very short broad neck with wide base and broad shoulders. Her weight was 25 pounds greater than average for her height. FSH (follicle stimulating hormone) assay was positive to 52 mouse units/day, which result is diagnostically significant of gonadal unresponsiveness to pituitary stimulation. A diagnosis of ovarian agenesis was made.

Two years after these initial endocrine studies were done, the newly introduced technique of chromosomal determination from skin biopsy was applied and a male chromosomal pattern was discovered. Thus the case is more correctly labeled gonadal agenesis and not ovarian agenesis.

The parents stated that the girl had always seemed a little small for her age but it was not until she was about 5 or 6 years old and starting school that they became concerned lest there were real stunting of growth. The parents themselves were relatively short individuals, both being 5 ft. 4 in. tall. The parents were also concerned by the girl's poor school performance. She failed first grade and had had to repeat. Her obesity had appeared only in the two years prior to her first examination at this clinic.

Estrogen therapy was begun when the girl was 14 yr. 5 mo. of age. Six months later it was noted in the endocrine clinic record that the breasts were developing satisfactorily, palpable breast tissue measuring 6 cm. in diameter. The areolae were erectile. Although pubic hair was still sparse, the labia minora and vaginal mucosa were well estrogenized. Her height on that occasion was 4½ feet.

Parents and family: The mother, who was the only parent available for interviews, was a tailored, placid sort of woman. Simple and sincere, she gave the impression that she did not get ruffled easily and had reared her family in an atmosphere of relative calm and tranquility. From what she reported, she had on occasion been strict, without being frenzied in her training of the children, especially in the case of the patient. She thought she had learned from her mistakes with the patient, and so was not quite so strict with the two younger children. She wondered whether some of the things she had done might have influenced the older girl's personality, and in doing some soul-searching she mentioned such things as having tied the girl on a leash to a clothesline at the age of three, to prevent her running away from home; as having tied her hands behind her back for some naughtiness which she could not recall (probably turning on the gas jets); and as having in general, used a lot of verbal do-and-don'ts.

The mother appeared thoughtfully concerned rather than agitatedly harassed about her daughter's personality at the time of the first interview. She said that she had never felt close to the child, who was never one to do much confiding. She had begun to think that

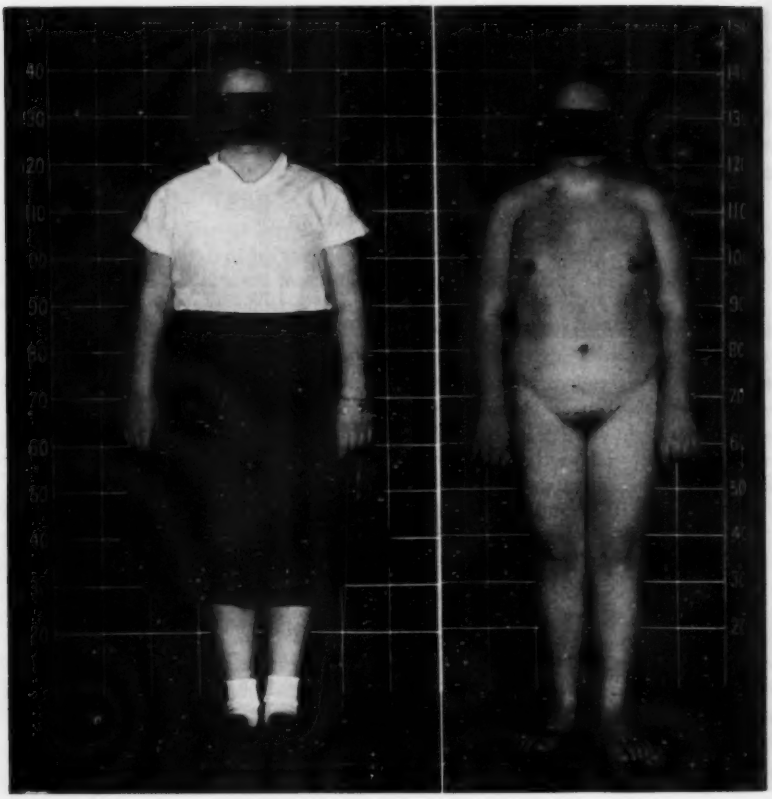

FIG. 1

the child must be unhappy because she seemed at times to be irritable, complaining and ungrateful.

The paternal grandmother, who was quite senile when she died, had lived in the house nearly all of Marilyn's[6] life. She was, according to her daughter-in-law, an irritable old lady, very difficult to get along with and rather childish. She had always been spiteful and unpleasant to Marilyn, as to everyone. The girl had, in the past been retaliative and had gotten into fights with the old lady, but she had gradually come to understand that her grandmother should be ignored and treated charitably—which was the mother's policy. The patient herself had nothing unkind to say about the grandmother, and gave no indication that she thought the old lady disliked her.

The girl's only comment when asked about her father was: "I get along with him okay; I don't do very much with him together. He belongs to a lodge and on Friday nights he has his regular meeting, and on Monday nights he has committee meetings and here, lately,

[6] The names of persons used in this case illustration are pseudonyms.

for two or three nights, he goes out and sells tickets on deep-freezes." Her father was manager of one of a chain of radio shops in the city. The girl was as noncommittal about her mother as her father: "She treats me nice. I get along with her okay. Sometimes I want to do things she doesn't want me to do."

Siblings: There were two siblings, Susan and Jerry, whose ages at the time of the first interviews were 10 and 3 years respectively. The mother commented that both girls adored their young brother, but that Jerry liked Susan better than Marilyn and often made clear his preference by refusing to let Marilyn do things for him. The mother felt that this was partly due to Marilyn's earlier clumsy handing of the boy. She was also more strict than Susan was with Jerry, and the boy resented it. The mother commented that Marilyn was unresponsive to advice and instruction. Uncertain as to whether Marilyn was obstinate about taking advice or simply slow to learn, she had found it difficult not to show favoritism despite her conscientious efforts to avoid it. On one occasion the girl told us very spontaneously: "Susan does a lot of things well, nearly everything better than me. It makes me mad. But there's one thing where I'm better than her. My skin's better. Susan is always playing with dogs, and gets fleas off the dogs, then she scratches the flea bites and she gets eczema on her legs. It doesn't look pretty when she goes skating."

The patient and her sister had always been very good friends. They still played and did things together such as going to the movies. The girls had their separate groups of friends, though, and sometimes each girl entertained her friends without the other sister being present. Each had learned that she was not wanted on such occasions, and did not belong among a group of girls of different age.

First Impression: When the girl was first seen for psychologic study at the age of 12, one year after the first endocrine studies, the investigator who first saw her wrote: "She is a very short child who strangely combines stolidity and lack of vivacity with pallid blonde coloring and lumpish appearance. From her appearance, her age is a complete mystery. She has somewhat puffy looking cheeks, although her complexion is clear and fine textured. On second glance, I realized that she had attractive, clear blue eyes and very pretty soft, wavy blond hair. She has the characteristic facial structure of many girls with ovarian agenesis which gives them their ageless look, and the chinlessness which makes their face seem top-heavy. Her manner on being greeted was polite, but without either enthusiasm or antagonism, or even curiosity. Her expression, though not by any means vacuous, was the expression of one who accepts whatever life presents without query or challenge."

Interviews: The child was seen on eight separate occasions between the ages of 11 yr. 5 mo. and 15 yr. 0 mo. Interviews and tests were conducted for a total of about ten hours. Each of the three interviewers saw her at least once.

As a general rule, the girl was friendly and cooperative in interviews, but as a responding and not an initiating participant—which was quite in keeping with the mother's descriptions of her social behavior. The flow of her conversation easily ran out, and needed prompting to keep it going. The one marked exception to this rule was in connection with reporting of her daydreams and fantasies: when the girl reported these fantasies, she was spontaneous, fluent, and much more candidly intimate.

Thumbnail self-picture: During the first interview, the girl dictated a description of herself in the guise of an introductory letter to a penfriend. With the interruptions of dialogue omitted, her self-picture was as follows: "Well I'd say I have short, pretty blond hair, blue eyes. Short, fat and about 4 feet. Let me see. I'm pretty well liked, and I don't know what else. I have a pretty good personality. Sometimes my moods are good and sometimes they're bad moods. The bad ones are mostly when my sister follows me all around. My good ones are when boys ask me to do, I mean tell me they like me or something like that.

I like house work. I like to play kick-ball a little, not too well. I don't like to write and I don't like to do arithmetic—spelling or reading are not too bad."

Day-to-day routines: The mother always was of the opinion that of her two daughters the older girl was the more difficult to understand. When the girl was first seen, the mother was concerned about her dreaminess. She said that at times it was even hard to get the girl's attention because of it. She wondered if it might affect her schoolwork.

In contrast with her sister, who discussed almost everything with her mother, the older girl was a quiet person not given to discussing her feelings with anyone. Relatives sometimes commented: "Marilyn's just like a little old woman, she sits there all grown up." Nonetheless, the mother felt that, while the girl did seem strangely mature in some ways, there were many ways in which she was still very childish. Even at 11½, she played with paper dolls and enjoyed playing house. On the other hand, she had never given her mother any serious concern about her ability to make and keep friends. In general she was a leader of the girls among whom she played. Characteristically, she organized the group into action, and then would stand on the sidelines as observer and supervisor rather than participant. The Girl Scout leader was impressed by the girl's ability to get things organized, and reported that she was well liked by the other girls.

Like many preadolescent girls, this girl had formed a devoted admiration for an older girl in the neighborhood. At the age of 12, she regarded 18-year-old Betty Jane as her very best girl friend and the person she admired most in all the world. Talking about Betty Jane, she said: "She'd rather be with me than she would with people her own age. She takes us to the drug store and buys us candy and things. Sometimes she takes us to the movies." For the girl, Betty Jane represented the epitome of teenage sophistication. She related with obvious delight how Betty Jane had met some sailors in the French navy and was planning to go to France that summer, ostensibly to learn the language, but secretly hoping she would meet the sailors again.

In addition to the older girl, there was another special girlfriend more nearly the child's own age. She said of her: "Mary is sort of funny, some of the other girls don't like Mary. They don't like her because she seems stupid sometimes. She asks the funniest questions." For another thing, "I sure would like to have Mary's figure, but I wouldn't want her hair and her eyes." Mary joined with her in playing house and pretending to be a movie star and to have radio programs.

Other of the girl's interests and pastimes were disclosed when she added in the letter to a penfriend, previously mentioned: "No hobbies. I like to collect silver, maybe. I play, and play around, and play records and things like that. On weekends I usually go to the Children's Theatre from ten to twelve and then I come home at one o'clock and go to the skating rink until five and come home and eat, watch television and go to bed. We're putting on a play—Alice in Wonderland. I'm the frog footman. I'm in Girl Scouts and that's about all. Oh, and I sing. Some people think I can."

The girl at 12½ did not like school. It was one of her wishes, on the three-wishes-test, to be grown up because, she confided, she really would like to quit school. She said she liked house work "ten times better than I like school." By the time she was 14, her former antipathy had changed and the girl had discovered that school was more to her liking than previously. She had just started junior high school, with an entirely new group of people. Undismayed at being plunged into an entirely new group of classmates, she was quietly confident about her ability to mix with people and make new friends. After the change to junior high school, the mother noticed a gradual change in the girl. She seemed less dreamy and was certainly applying herself better in school. During the summer months the girl had gotten more active in sports was enjoying playing all kinds of ball games and had been

doing a great deal more roller skating. Her mother considered that she was keeping up with the rest of the adolescent group in her changing tastes, clothes preferences, mannerisms, and so forth.

The girl gave no evidence of special talents or abilities to exploit. She liked to sing, and fancied that she would like a singing career. Almost taking her fancy seriously, she conjectured that she would have to try for an operatic career for, though she preferred popular music, she had a high voice and opera stars all seemed to have high voices. Once a neighbor told her that she was another Kate Smith, and she nearly burst with pride. Her daydreams of ambition and success, included one of being an opera star who would go on the stage and give a wonderful performance. The audience would shower her with applause and call her back for bow after bow. She would be handed great bouquets of flowers.

Intelligence test: The Wechsler Intelligence Scale for Children was administered when the girl was 12 yr. 4 mo. old. She achieved a rating of dull normal: verbal IQ 94; nonverbal IQ 82; full scale 87.

Body morphology and appearance: At the time of the first interview, the girl was not entirely sure what all the medical examinations were about, but she thought it was because of her shortness. She knew that she was short—much shorter than any of the other girls her age. She had come to take it very philosophically, thinking that maybe she was just intended to be short like her father. When she'd asked her mother about it her mother had said: "It's not anything, I guess you're just naturally short." Despite her apparent acceptance of her shortness, there were other indications that her stature was, nevertheless, one of the things about herself that she would change if she could. Her first prompt response to a request to make three wishes was: "I'd like to get thinner—and taller," (almost as an afterthought). She said that she thought she wouldn't mind taking pills to make her grow but, if it required an operation, her size didn't really make that much difference to her.

By the time she was 15, she was very nearly 4½ feet tall. She remarked that she was not too unhappy about her size, and that if she could reach 4½ feet she would be reasonably satisfied.

From the outset she had been given an understanding of what treatment would ultimately accomplish, and of the desirability of not terminating bone growth prematurely with estrogen therapy. Although there was no doubt but that the girl was looking forward with pleasure to ultimate breast development, she was never impatient for treatment to begin. As far a she had been able to observe, very few of the girls in her class at school had breast development, and she did not feel an outsider because she had not yet begun to develop. She was content in the knowledge that if she grew really impatient for treatment it could be commenced without further delay. Estrogen therapy was begun at 14 yr. 5 mo. When interviewed just before her fifteenth birthday, the girl gave unequivocal evidence of her pleasure in the breast changes that had occurred.

Sexuality: The girl's parents, like many parents, had been dilatory in family discussion of sexual matters. They had assumed it would be quite soon enough to speak of menstruation at puberty, and of the details of conception and childbirth when their daughters got to the dating age. Thus when the girl was twelve she had been given a book to read on the facts of menstruation. During the early teenage, when the mother finally tried to speak to her daughter about sex facts, the girl had giggled with embarassment and peremptorily dismissed her mother, saying that she knew all about such things. To the investigators, it became apparent when the girl was seen at the age of fifteen, that there was still considerable ignorance and frank distortion in her stock of sex information and that a straightforward discussion of the entire subject was in order. Presented in this manner and with the use of anatomical diagrams, the girl was interested and learned readily.

By the age of 11, the girl had established a strong sense of propriety with respect to the rightness and wrongness of erotic play with boys. Although she had seen her little brother naked, there were no instances of sexual inspection or play with boys, so far as could be determined. A furtive kiss at parties was a real adventure for her. She told one interviewer that Rudy, a sixth grader who lived just down the street from her home, was her boyfriend. With a note of regret in her voice, she added that she didn't think she was Rudy's only girlfriend; there seemed to be other girls interested in Rudy. She went on more cheerfully, saying brightly that, in any case, she thought she really liked fifth-grader Billy better. The interviewer inquired in lighter vein whether she went steady with any of her boyfriends. She giggled girlishly and said: "Oh, no!", hastening to add, "but if I could get one I would."

In connection with her daydreams of being an actress, the girl also contemplated marriage: "I'll want to get married and have two children. I'll call the boy Billy—I'd like a girl and a boy—I'd call the girl Kathleen." On another occasion she envisioned that in ten years: "I'd probably be married. I'd be working and have a maid. My husband, he'd be 25, no 24, no I mean that he's 25 and his hair's blond, it's sort of wavy and curly. He earns eighty dollars a week and I'd earn seventy dollars a week." She went on, in response to a query about what sort of wedding she'd have had, to say: "A big wedding—I had on a real pretty white dress and he had on a blue suit. It was white satin trimmed with pearls and a veil, and a longer veil at the back, and it would have a white beanie sort of hat, and long white gloves and white carnations."

Although the girl was unable to remember any of her dreams, she did recall several of her fantasies. As is not uncommon among girls of her age, some of her fantasies were erotically masochistic in character. Reported during one of the early interviews, these masochistic fantasies always occurred in the bedtime period before going to sleep, and involved murders, torturings, beatings and so on. In them the girl herself was being murdered. The murderers were usually "a bunch of men." Only rarely were women among the members of the fantasy murder gangs. Sometimes instead of murdering her outright, the men would torture her, the girl said. Typical of these fantasies was one in which she was walking down a street going quietly about her business when, suddenly, she was beset by a group of people who forced her at gunpoint to go with them, into the country, where her cries wouldn't be heard; once there she fantasied being murdered, beaten and hurt. Questioning revealed that in the torture fantasies she had her clothes torn off, was cut with knives, was beaten with a club, or had various things stuck into her vagina, including knives and men's genitals. Sometimes she would get an accompanying feeling of excitement. It was experienced primarily as genital excitement. It was most intense during the parts of a fantasy where she was being tortured. She considered the genital penetration episodes of the fantasies less exciting than the ones where she was hurt. Manual or thigh-pressing masturbatory activity was said to accompany these fantasies often, though not invariably. Masturbation was also, at times, carried out in the absence of fantasy. From what the girl said, the erotic excitement accompanying her fantasies was indistinguishable from the sensations produced by touching or pressing the genital area. She reported no evidence of orgasm.

Illustrative of some of the girl's romantic Prince-Charming fantasies is the following story which she composed for card 3 GF of the Thematic Apperception Test:

> Once upon a time there was this girl and she had this boyfriend and she liked him a whole lot and he liked her. They were going to get married but one day her boyfriend went away and they couldn't get married so she started to cry and everything. This is where the picture comes in. Then finally at the end her boyfriend comes back and they get married. Well, they live very happy.

The encroachment of adolescent sophistication was also evident. She composed the following story for TAT card 6 GF:

> This lady was sitting on the couch or sitting down on a chair one day and her husband came up to her and he said, "you've been going around with another man and I don't like it so I'm going to New York, I mean, I'm going to leave you." So he decided and he went and left her and he went to New York. And so he went to New York. The lady, they found out, he found out that she really and truly was going around with another man. Finally at the end he decided to come back and so he came back and everybody was very happy.

Although the taboos of rearing served to inhibit heterosexual erotic play, the girl, like many prepubertal girls, had been involved in some homoerotic games around the age of 11. These games were with a playmate already beginning puberty. The girls often visited one another after school and sometimes, when the mother was out of the house, they would take off their clothes, get into bed together and tickle one another's genitals. The girl was quite mystified that her friend seemed to enjoy having her breasts fondled. In reporting the experience, the girl intimated that it had been a very secret sort of play fraught with a certain amount of guilt. Subsequently there had been some sporadic genital play with her juvenile sister, with whom she regularly shared a bed; it was erotically unrewarding and was soon discontinued.

By the time the girl was 15, it was only after a specific reminder that she recalled ever having had the masochistic fantasies she had formerly related. Daydreams and fantasies of romantic courtship had, on the other hand, proliferated and been embellished. More often than not the central figure in the fantasies was one or another of the boys at school whom she admired from afar. Sometimes she fantasied that 15-year-old Jerry would come to her home bringing a corsage and, dressed in a long evening gown, she would go with him to a big school dance. Sometimes she fantasied that after the dance Jerry would take her home and hold her hand. At other times, the fantasy would include a stopover for a soda, and the glorious evening would end with a goodnight kiss at the doorstep of her home. With a blush, she said that on occasions she thought of Jerry touching her breasts—that was especially exciting, but she scarcely dared to imagine him touching her genitals.

Although her fantasies at times led up to masturbation, she considered it far more sexually exciting to dance very close to a boy at a school party. Then she would become aware of increased vaginal secretion. The girl herself felt certain that her genital erotic sensations had been markedly enhanced by estrogen treatment. From what she said, it was difficult to tell whether she had experienced orgasm or not.

At 15, the homoerotic play of former days had long since been abandoned. From her own statement, there had been no homoerotic games since she was 11. A few whispered stories of lesbianism heard at school she considered "not very nice." On the other hand she admitted that, when she saw sexy magazine pictures of pinup girls, the possibility of homoerotic play crossed her mind. Simultaneously, however, the sexy feelings engendered in her by these pictures became associated with the still rather taboo idea of erotic play with a boy. Contemplating the theoretical possibility of being seduced by a girl she supposed she would just walk away and say: "No, I think you're crazy."

In contrast with the romance and eroticism of her daydreams, her romantic attachments were rather stilted, restrained and prudish. Nearly every week she attended a dancing class and went to all of the school parties and dances of her school class. Sometimes she went with a boy but more often, like many of her contemporaries, found dancing partners from the stag line. Her youngster sister was a great favorite with all the boys. The mother disclosed

that she had been rather concerned by a story "from the grapevine" that Marilyn was more interested in the older male dancing instructor than in the younger schoolboys. She also disclosed that Marilyn had been rather prankishly fond of a very fatherly married man who frequently visited the home with his wife. The mother was made uneasy and confused by these things. The investigators interpreted such behavior to signify that the girl had not yet transcended the taboos of her upbringing as successfully as her sister had and that, in an inchoate way she was, so to speak, playing safe with an older and fatherly man.

There were important changes in the girl's general behavior that occurred in the wake of the puberty changes induced with estrogen treatment. The former don't-care attitude that had so colored her earlier school experience had disappeared. The teachers were satisfied with her work and she seemed to enjoy school. The girl had, in fact, been quite upset at having to miss school in order to come to the hospital; it was the only day she had missed from school the entire year. In social activities she was participating in early teenage affairs, only slightly in arrears of her age group, with a great deal of interest and enthusiasm. She was very much a leader and enjoyed planning parties. Though the mother continued to have little access to the girl's confidence she was no longer worried about her daughter's daydreaminess. The girl now busied herself with her studies, her personal laundry and so forth, instead of moping as before.

Appraisal: The special significance of this patient is twofold. First, she had unquestioningly been reared as a girl and at the age of 12 was diagnosed as a case of ovarian agenesis. By the time she was 14, a new technique of chromosomal sex determination had been developed, and only then was it discovered that she had male chromosomes and gonadal, not ovarian, agenesis. Second, she was interviewed over a period of three years when the onset of puberty usually occurs in girls, but she had none of the physical signs of puberty which accompany estrinization, owing to her gonadal condition.

Despite sexual infantilism physically, the girl was not infantile psychologically. On the contrary, her sexual psychological development had progressed along lines typical in many girls of her age. She reported several extremely masochistic fantasies of being beaten, mutilated, sexually assaulted and even murdered by men. These fantasies were accompanied by heightened erotic sensation in the genital organs, along with manual or thigh-pressing masturbation; they occurred chiefly in the bedtime period before going to sleep.

Apart from these masturbation fantasies of feminine subjugation, the girl reported another group of romantic courtship and marriage dreams which occurred as daydreams during waking hours, as well as at bedtime. In her TAT stories, 3 out of 10 were tales with a similar theme. The girl's professed ambitions also included an unequivocal anticipation of getting married and having two children. Some of her daydreams were woven round boys of her acquaintance, but she had not declared her amorous feelings toward them in person.

So far as dating was concerned, the girl had not joined to any significant degree in the social and dating activities of her contemporaries. This lag in her development was associated partly with her IQ rating of dull normal and with the fact that she had failed two grades in school and mixed mostly with children younger than herself. The lag was also associated, and more significantly so, with her juvenile body morphology and immature appearance. She was not sexually attractive to adolescent boys, and could not keep fully in step with her age-mates. In romantic affairs the only avenue completely accessible to her was that of daydream and fantasy.

With regard to childhood sexual play and experimentation, the girl reported that she had had no experiences with boys and indicated that such possibilities were morally vetoed. She had, however, at the age of between 10 and 12, been engaged in sexual play with an adolescent girl friend on a few occasions. Subsequently there had been some more, but less

thorough sexual play with her sister with whom she regularly shared a bed. The girl enjoyed the erotic clitoral sensations stimulated in these play practices, but she was quite mystified that the adolescent girl enjoyed having her breasts fondled.

The episodes of homosexual play were the only evidence which one may use in support of a theory that this girl's male chromosomes had some determining influence on her sexual outlook and orientation. Three considerations militate against such a theory. First, all the evidence of her deportment and demeanor, her off-guard comments, her professed ambitions, and her daydreams and fantasies all pointed to a strongly feminine outlook. Second, she was the only patient among 11 with gonadal agenesis and male chromosomes who reported homosexual eroticism of any sort. Third, it has been well established in personality investigation that as many as one out of three heterosexual women report having had transient homosexual episodes in the early teenage (7).

It is quite likely that transient homosexual experiences in early teenage are a sequel to, as well as a source of sexual perplexities and confusion. There was evidence that this girl's rearing engendered bashfulness, shame and guilt concerning sexual things, and paved the way for a series of misconceptions about the vulnerability of womanhood. In any case, her homosexual play was part of her early teenage negotiation of sexuality. It was a sign that she was having her fair share of difficulties in making this negotiation.

With regard to the outcome of this negotiation, the long term study of this girl was particularly instructive in giving evidence of the development of feminine sexual orientation and outlook in a girl who, in childhood, had been reared with rigid taboos on sexuality of any sort and, in preteenage, had had transient episodes of homosexual experience. Three developmental changes can be distinguished:

1. In the first place the girl's masochistic fantasies of sexual mutilation at the hands of brutal men had been displaced by a gradual lifting of taboos on romantic courtship fantasies involving uncensored imagery of erotic play with boys.

2. A taboo on homosexual play had been strengthened, and overt homosexual play disappeared altogether. Likewise homosexual fantasy disappeared. The only remnant of homosexual orientation that remained was fleeting imagery specifically associated with the viewing of magazine pictures of pinup girls. Yet, the homoerotic possibilities which these pictures evoked were simultaneously accompanied by imagery strongly oriented toward erotic play with a boy. In this respect the girl was like many women who are erotically aroused by pictures of pinup girls, but always so that they identify with the figure in the picture and imagine themselves being seductive and erotically appealing to men. Unless one is cognizant of this phenomenon in normal women, one can easily be misled into false conjectures about latent homosexuality.

3. Finally the girl had become more adventuresome with dating, and enjoyed untrammelled sexual feelings while dancing with a boy. At the age of 15, however, she had not lifted every last vestige of taboo on eroticism with boy friends. One thing in particular revealed a residue of earlier strict taboos. This was the fondness she had for two older married men with whom she playfully coquetted in an apparent effort to enjoy physical contact. The investigators considered this behavior to signify that the girl had not yet transcended the taboos of her upbringing as successfully as her sister had done, and that in an inchoate way she was, so to speak, playing safe with an older and fatherly man. In psychoanalytic terminology, this behavior might also be viewed as Oedipal in significance, in which respect the finding of a male chromosomal pattern in this girl is most pertinent and elucidative. Quite obviously, it is impossible to attribute such feminine Oedipal manifestations to chromosomal determinants in this chromosomally male person.

Among the indisputably recognizable sources of teenage difficulty with which the girl was

confronted, one was her sexually infantile appearance, during the early teens. Another was her physical uncomeliness which was not helped any by her stocky stature, despite her own acceptance of shortness as a familial trait. Yet another was her grandmother, an irrascible and unreasonable old lady, who had a special dislike of this granddaughter with whose family she had lived for years, until she died.

The girl's repertoire of methods and modes of dealing with the difficulties and stresses in her life utilized chiefly some form of constraint or inhibition. She retired into the social background and into the solitude of personal privacy, rather than engage in aggressive strategies or insistent mastery. In social contacts, she appeared a taciturn, rather pallid personality, hard to get to know and difficult to feel close to; she was cautious about letting her guard down in candid and confiding intimacy. At home she was so reserved and daydreamy that her mother had at one time wondered whether she were dull or lazy, or unhappy and secretly worried. At school the girl resigned herself to failures and betrayed anxiety only in a vestigial snigger. She shrugged off the grandmother's bickering and meanness as a mere nothing, her retaliatory resentment showing itself circuitously and unplanned as disdain. Resentment and aggressiveness also showed itself sometimes in unstrategic displays of truculent obstinacy. An emphasis on control was evidenced in the Rorschach Test in the literal-minded and guarded way in which she picked segments of the blots, and gave one response to each card.

None of the three investigators was prepared to evaluate the girl as neurotic, nor as psychologically disturbed to a significant degree, despite the too frequent operations of various modalities of control and inhibition. The signs of personality which she manifested were evaluated rather as marked personality traits of a type frequently enough encountered in so-called normal people, especially in adolescents. There was no evidence that the girl would undergo any radical personality changes in the future, either for better or for worse. She was negotiating the ups and downs of her life in her quiet, yielding, unresistive way and seemed likely to continue to do so for a long time to come.

GENERAL FINDINGS

In all, 11 patients with male chromosomes and gonadal agenesis have thus far been studied. Since no anomaly of the female-appearing external genitalia was present at birth, all were reared as girls. They have ranged in age between 9 and 27 years at the time of study.

Gender Role and Sexuality

Unequivocal femininity was an outstanding feature of all of the 11 patients. It can be stated with conviction that in not one single instance was any doubt raised, by any physician who saw these patients, concerning their thoroughgoing femininity. Further, so far as is known, not once in medical history has anyone ever suggested that persons with this condition should change to live as boys. In all respects these individuals fulfilled the cultural and psychological expectations of femininity so completely that it was, in each instance, impossible to envisage the person as a boy or man. All the data collected from these patients contained nothing that would effectively contradict the foregoing statement, and there was an abundance of detailed data to support it.

Table I summarizes the data with respect to the erotic fantasies and practices of these patients.

TABLE I

Erotic Orientation in 11 Patients with Gonadal Agenesis and Male Chromosomes

Erotic Orientation	In Childhood or Before Treatment (8 cases)*			In Teenage or Adulthood After Treatment (3 cases)*		
	Yes	No	Inconclusive data	Yes	No	Inconclusive data
Daydreams and fantasies of courtship, marriage and erotic play	5	1	2	3	0	2
Genital practices and erotic stimulation	1	5	2	5	0	0
Homosexual eroticism of any sort	1	5	2	0	5	0

* 2 cases studied before and after treatment.

First, concerning daydreams and fantasies of courtship, marriage and erotic play, 5 of the 8 younger patients before treatment and 3 of the 5 older patients after treatment gave positive evidence of having had such daydreams and fantasies; none gave negative evidence. It is clear that, in mid-childhood and later, these girls and women with male chromosomal patterns were just as likely as their contemporaries with female chromosomal patterns to have daydreams and fantasies of romantic courtship, marriage and erotic play.

Second, concerning genital practices and erotic stimulation, all 5 of the older patients gave positive evidence of genital practices (either heterosexual intercourse, or more usually, masturbation), whereas only 1 of the 8 younger patients gave positive evidence, while 5 gave negative and 2 gave inconclusive evidence. The relationship between genital sensation accompanied by erotic imagery, on the one hand and estrinization of the genital tract, on the other, is difficult to appraise. All of the estrinized patients reported genital sensation, but only two of them connected it also with erotic imagery as in daydreaming and fantasy. By contrast, only 1 juvenile patient reported genital sensation and she connected it definitely with vivid erotic fantasies, as well as with manual or thigh-pressing masturbation. We conjecture tentatively that estrinization is not a prerequisite of genital sensation and erotic imagery, but it is a strong catalyst, so to speak. Estrinization is prerequisite of orgasm in the female insofar as it heralds the growth of the vagina to full adult size. None of our patients was experienced enough in genital practices to give relevant information about orgasm. Four of the patients, however, voluntarily discontinued estrogen therapy for intermittent periods of months or years, in the late teens or early twenties, and uniformly reported no subsequent change or impairment of sexual feelings or erotic imagery.

Third, concerning homoeroticism of any sort, none of the older patients and only 1 of the 8 younger patients gave positive evidence of homosexual orientation or activity. In that one patient, some preteenage episodes of homosexual

play were reported. Their significance has been fully discussed in the appraisal section of the case illustration.

All in all, the presence of male chromosomes in these eleven girls did not in any way distinguish them from eleven girls selected randomly except for one consideration, namely that the absence of hormonal puberty, until its artificial inducement with estrogen substitution therapy, in most instances proved to engender various impediments to psychologic and social maturation. The chief of these impediments was a juvenile stature and appearance.

The psychologic concomitants of small stature and juvenile appearance in physical sexual infantilism are, in themselves, important matters on which these cases throw light.

Stature

Among the many physical anomalies found at times in these patients the one shared by all of them is shortness of stature. The tallest of the patients in the present series was only 58 inches, a height which Wilkins considers to represent about the maximum attained by patients with this condition. A far greater number of patients do not even approach this figure, and a final height of between 52 and 55 inches is modal. In other words, most of these patients, when full grown, have the height of the average ten or eleven year old girl. It is not surprising that, more than anything else, these girls and their parents were initially concerned about their shortness of stature.

While none of the patients had the excruciating problems related to size that are to be found in other types of more severe dwarfism, shortness of stature was nevertheless an important element in shaping the personality of these people. To say this is not to imply that these girls and women were psychologically marred because of their size, but rather that their size gave a special coloring to the personality because of the way in which they were treated by people about them. If the girl was noticeably shorter than her agemates in the earliest years of childhood, she was able, more often than not, to exploit her petiteness in a way that people consider cute, and so negotiate the problem. This maneuver did not, however, permanently compensate for the disadvantages of shortness. As they grew older, the short girls bolstered their self-esteem for a time by an implicit expectation that sooner or later they would catch up in their development. Eventually, in the teenage, if not before, each girl had finally to face the fact that her permanent lot in life was to be unusually short. There was considerable diversity amongst the girls as to how they faced up to this fact. None was completely crushed, and defeated in some sort of yielding self-effacement. One in particular reacted with rather futile, compensatory efforts of mastery that showed up chiefly as an inability to get along with people, through finding fault with them too often, and subsequently deprecating herself. This girl's shortness was silently resented by her parents, a

factor which was undoubtedly a potent influence on the child's own reactions to her stature. The majority of the girls, by contrast, evolved serviceable ways of negotiating the various indignities of shortness, sometimes with a touch of haughtiness, sometimes with bantering and a joke.

Infantilism

The transition from prepuberty to adolescence is a gradual one which evolves in the setting of pubertal physical changes and of social interaction. A child's anticipation of being grown up and of being considered grown up by others directs her attention to the morphology of her body. Above all others, the physical signs of puberty changes are the signs which signify to other people that a girl is now ready for the social transactions of adolescence. Thus, the prepubertal girl usually anticipates eagerly the first signs of breast development in herself, and compares her own progress with that of her agemates. In a way which enhances the girl's sense of confidence in her own femininity, the menses are a private sign that she is like all others of her sex.

Without estrogen replacement therapy, girls with gonadal agenesis remain infantile. Upon institution of therapy, there is rapid growth of the breasts, labia and vagina, estrinization of the vaginal mucosa and development of the uterus and tubes. Cyclic therapy, with lowering in estrogen level every fourth week, permits endometrial breakdown and menstrual bleeding.

In the present series of 11 cases there were only 2 in whom diagnosis and treatment were delayed beyond 14 years. These two, and also two others[7] amongst the nineteen, were the only ones who were unable to keep in step with their contemporaries in regard to general teenage social and psychological maturation. Their juvenile appearance had militated too strongly against them during these critical years of their life, and the subsequent process of catching up, once out of step with their contemporaries, was a slow and tedious one. All the other patients who began with substitution therapy before the age of 14 were able to keep up with their agemates quite successfully. Resembling other adolescent girls, they found their social eligibility greatly enhanced. Having laid the cornerstones for future womanhood in childhood daydreams and fantasies of courtship, marriage and erotic play, they now had obtained the pass-key to those life experiences in which daydreams become translated into a reality that had been previously inaccessible.

In the overall view, these cases of gonadal agenesis demonstrate very clearly that the appearance of a physically mature body is an essential ingredient for the advancement of psychological maturation in the teenage. If juvenile appearance persists beyond the middle teens, the task of psychologic advancement is so great that it can be successfully negotiated by only the exceptional person.

[7] One of these two had female chromosomal pattern; a biopsy of the other was unobtainable.

Intelligence Quotient

It has been said that ovarian agenesis is associated with congenital impairment of intelligence. The IQ ratings of 16 of our cases of ovarian and gonadal agenesis are graphically presented in Figure 2. Despite the smallness of our sample, the data show conclusively that a low IQ is not an invariable concomitant of the condition and that, on the contrary, some patients gain ratings at the highest level.

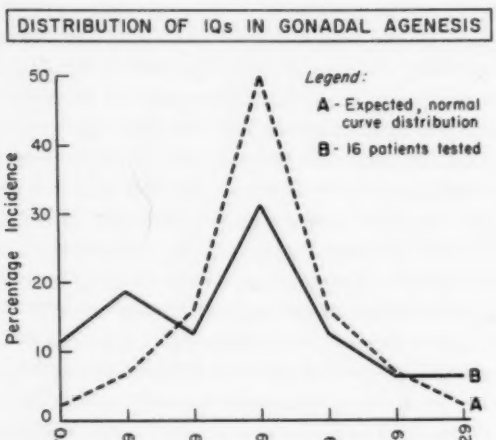

FIG. 2

Personality Healthiness

We rated these patients on a 4 point scale of personality healthiness and nonhealthiness. The four points were:[8]

Healthy
Mildly nonhealthy
Moderately nonhealthy
Severely (morbidly) nonhealthy

It was evident, beyond all shadow of doubt, that none of these patients was severely and morbidly nonhealthy. None was appraised as psychotic, and no physician, psychiatrist or nonpsychiatrist, could possibly disagree.

[8] Neurotic symptoms, though they may be either mild or severe, for the most part require a person to be rated as *moderately nonhealthy*. Psychotic symptoms though at times only mild or moderate, usually require a person to be rated in the category of *severely or morbidly nonhealthy*.

There was only one patient for whom the degree of nonhealthiness required a rating as much as moderately nonhealthy The nonhealthy signs which she displayed were, according to current nosology, more in the nature of a mild adolescent maladjustment problem or a mild personality trait disorder than a classically neurotic reaction. It was legitimately debatable whether it was being overscrupulous to appraise this girl as moderately nonhealthy; perhaps she should have been rated only mildly nonhealthy.

The remaining ten patients were rated as healthy in personality functioning. To rate a person healthy is not to deny that they have, or have had problems to cope with. Nor is it to deny that their personalities in some way bear the imprint of their problem encounters. In the present instances, this imprint was in the nature of a uniqueness of personality traits which gives a person his individuality and ordinarily is recognizable as one of the many variations of the so-called normal. It was possible, of course, to decipher psychodynamic functioning in the personality of these healthy people as in all healthy personalities. Psychodynamic functioning is not the special prerogative of the nonhealthy personality.

As was indicated in the foregoing section, the common problem that confronted these girls was their permanently short stature and their infantile appearance before treatment; for each girl this was the most baffling and taxing problem also. It became quite evident to the investigators that it was the most socially obvious of the stigmata of gonadal agenesis that engendered the most distressing and ramifying difficulties for these girls. Thus infantilism engendered more problems than webbed neck, webbed neck more than lack of menses and lack of menses more than sterility. It was in fact very surprising that the girls showed such lasting equanimity in coming to terms with the knowledge that they would have to adopt their children. Cardiac invalidism, another of the possible stigmata of the syndrome, was not severe in any of our cases.

The commonest way these patients negotiated the feeling that they were slightly freakish, was in the overuse of inhibitory constraint and a reciprocal lack of mastery, aggressiveness and initiatory verve. Five of the girls in particular appeared phlegmatic and rather stolid. They seemed to live well guarded within the confines of their personal inviolacy. Their personal confidence was rather inaccessible and they were talkative in a friendly but quite taciturn way. One other girl had gone untreated for such a long time that she remained dallying in psychological infancy until the age of 17, but at 23 was much like the girls described above except for being much more infantile, incompetent and more unself-assured. There was only one other girl who had gone so long untreated. When she was seen at the age of 26, the chief manifestations of inhibition and control function in her personality were in the nature of strict conformity to moral and moralizing rules by which she regulated her life. She was an animated, vivacious person, whose self-sacrificing dedication to

humanitarian service could be characterized as cheerful missionary zeal rather than martyrdom.

The remaining two of the eleven patients did not show any special intensity of inhibitory control nor excessive manifestations of mastery and acting out. They had achieved a serviceable balance between inhibitory and mastery functions of the personality so that one observed no evidence of exceptional ways of coming to terms with the stigmata of gonadal agenesis.

GUIDE TO CASE MANAGEMENT

On the basis of experience during 3½ years with nineteen patients, girls with gonadal agenesis and either male or female chromosomes, the following general guide to psychologic management has been formulated.

1. Girls clinically diagnosed as having gonadal agenesis benefit by being told fully of the reasons for repeated genital examinations. Frequently a girl will have been told only that she has been brought to the hospital because of short stature; under such circumstances she may be bewildered by detailed examinations of the genitals.

2. As soon as the diagnosis has been fully established, girls with gonadal agenesis, and their parents as well, profit from being told simply and straightforwardly the medical facts of their condition (excepting data on chromosomal status—see recommendation 3). By such frankness a girl's conjectures and private fears of being destined perhaps to be a freak or a midget are allayed, and other possibly morbid speculations are forestalled. An accurate knowledge of the basic elementary anatomy and physiology of the female reproductive system is a prerequisite to a girl's understanding her own special deviations from normalcy. Acquaintance with proper genital vocabulary helps both the girl and her parents to talk straightforwardly about the condition. Simple diagrams of the internal reproductive organs have proven useful in disabusing some patients and their parents of the notion that there is "nothing at all inside". That the developmental deficit is specifically of the gonads, rather than of the accessory reproductive structures, is important information in reassuring the girl and her parents that her infertility need by no means deprive her of the erotic satisfactions of marriage.

3. Girls with gonadal agenesis and male chromosomes are benefited if they remain uninformed of their chromosomal status. Should they obtain this information, they may be, unless specifically disabused, unnecessarily confused and influenced by present day misconceptions that the entirety of one's sexuality is genetically determined. If there is one salient finding that has emerged from the present study, it is the finding that a person's conviction of himself as a man or herself as a woman—the gender role and erotic orientation—is a variable quite independent of genes and chromosomes.

4. As soon as the diagnosis has been fully established, girls with gonadal agenesis should be frankly informed of treatment possibilities. If it is desirable to postpone substitution therapy until the patient has achieved greater prepubertal height, and if she is already in the teenage, the decision concerning this postponement should be reached in consultation with the girl herself. She should understand the reason for the delay, and know that, should it prove intolerable, she herself will help to decide the date when to begin treatment. Taken into confidence in this way, girls have been able to tolerate a deferment for as long as two years. They have been quietly reassured by knowing what eventually to expect from treatment—by knowing specifically what physical changes treatment will bring about, and even by knowing such trivial details as that medication will be oral or intramuscular. While the favorable potentialities of hormonal treatment may be stressed, its limitations should be discussed with similarly unhampered straightforwardness. There should, for instance, be an assurance that a growth spurt of a few inches is to be expected during the early phases of treatment, but there should be an equally clear understanding that the most hopeful therapeutic outcome will be an eventual height of under five feet.

5. Girls with gonadal agenesis are benefited if they learn of their infertility at the same time as they learn of the possibilities for thoroughly adequate secondary sexual development. Some parents have suspected dire consequences should their daughter not be protected from the knowledge of her infertility until late teenage or even until marriage is seriously contemplated. Such parental misgivings are not justified. On the contrary, a girl can in early teenage or earlier assimilate the fact of her infertility with considerable detachment, as almost academic information. In this early period of her life, her adolescent daydreams are still far from being translated into actual experience, and her fantasies of childbearing are nascent. Well informed at this age, she can grow up better able to accept with equanimity her destiny of adoptive motherhood rather than of biological maternity.

SUMMARY

Nineteen girls and women with gonadal agenesis have come under psychologic investigation. This paper was concerned particularly with 11 of the patients who, among 13 whose chromosomal pattern has been determined, were found to be chromosomally male. All 11 of the patients were found unequivocally to fulfill the cultural and psychological expectations of feminity. The salient finding to emerge from the study was that a person's conviction of himself as a man or herself as a woman—the gender role and erotic orientation—is a variable quite independent of genes and chromosomes. In addition, these cases demonstrated very clearly that the appearance of a physically mature

body is an essential ingredient for the advancement of psychological maturation into the teenage. It was the socially obvious of the stigmata of gonadal agenesis that engendered the most distressing and ramifying difficulties for these girls. Of the patients studied, none was appraised to be neurotic or psychotic, although, in all of them their personality in some way bore the imprint of their problem-encounters. The commonest modality of personality function that these patients relied upon was the overuse of inhibitory constraint and a reciprocal lack of mastery, aggressiveness and initiatory verve. A low IQ was found not to be an invariable concomitant of the condition; some patients gained ratings at the highest level. A guide to case management was presented in which it was stressed that these patients benefit from a straightforward discussion of the medical facts of their condition.

REFERENCES

1. Albright, F., Smith, P. H. and Fraser, R.: A syndrome characterized by primary ovarian insufficiency and decreased stature; a report of 11 cases with a digression on hormone control of axillary and pubic hair. Am. J. Med. Sc., 1942, **204:** 625.
2. Moore, K. L., Graham, M. A. and Barr, M. L.: The detection of chromosomal sex in hermaphrodites from a skin biopsy. Surg., Gynec. and Obst., 1953, **96:** 641.
3. Grumbach, M. M., Van Wyk, J. and Wilkins, L.: Chromosomal sex in gonadal dysgenesis (ovarian agenesis): Relationship to male pseudohermaphroditism and theories of human sex differentiation. J. Clin. Endocr. and Metab., 1955. In press.
4. Jost, A.: Recherches sur la différenciation sexuelle de l'embryon de lapin, troisième partie: Role des gonades foetales dans la différenciation sexuelle somatique. Arch d'Anat. Micr. et de Morph. Exp., 1947, **36:** 271.
5. Jost, A.: Problems of fetal endocrinology; the gonadal and hypophyseal hormones. In: *Recent Progress in Hormone Research*, Edited by G. Pincus, Academic Press, New York, N. Y., 1953.
6. Money, J. and Hampson, J. G.: Idiopathic sexual precocity in the male., Psychosom. Med., 1955, **17:** 1.
7. Kinsey, A. C., Pomeroy, W. B., Martin, C. E. and Gebhard, P. H.: *Sexual Behavior in the Human Female*, W. B. Saunders Co., Philadelphia, Pa., 1953.

ROUS SARCOMA VIRUS INFECTION IN THE CHICK EMBRYO

I. Pathogenesis of the Rous Sarcoma Virus Infection in the Chick Embryo with Particular Emphasis on the Hemorrhagic Disease

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INTRODUCTION

Hemorrhagic lesions in chickens with Rous sarcomas were first described in 1913 by Rous and Murphy (1). Among subsequent studies (2, 3) of this hemorrhagic disease was a report by Duran-Reynals (4) of a higher incidence of lesions in young chickens than in old. The resistance of older birds was attributed to "virus-suppressing" antibody in their blood. Milford and Duran-Reynals (5) inoculated the virus intravenously and intracoelomically into chick embryos and produced only hemorrhagic disease, not tumors, in the embryos proper, an occasional small tumorous nodule being produced on the chorio-allantoic membrane at the site of needle puncture. The lesions in the embryos showed no neoplasia, but there was evidence that the hemorrhages might be due to destruction of endothelial cells. Virus inoculated on the membrane also produced hemorrhagic disease (6, 7).

None of these studies indicated whether the lesions were consistently produced, or how consistently. The only quantitative observation (5) stated that of 132 embryos injected with virus, only about 50 per cent developed the disease. If lesions were consistently produceable, it would seem to us that they would provide a convenient end point with which to measure virus activity. Therefore, in order to try to evaluate their use in this way, some of the factors influencing the development of hemorrhagic lesions were studied.

MATERIALS AND METHODS

1. Source of Rous sarcoma virus: Four types of virus containing materials have been employed.
   a. Sarcoma tissues from adult chickens, which had been inoculated with the virus, were harvested aseptically and stored in lusteroid tubes at dry ice temperature (−78°C) until used. The original strain of virus was obtained from Dr. W. R. Bryan of the National Cancer Institute in 1950.
   b. Chorio-allantoic membrane tumors were produced by the inoculation of either an extract of a tumor or a graft itself onto the chorio-allantoic membrane. They were harvested aseptically and stored in lusteroid tubes at dry ice temperature (−78°C), until used.

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c. Chick embryo livers with hemorrhagic lesions were harvested aseptically and stored in lusteroid tubes at dry ice temperature (−78°C) until used. In order to determine whether the hemorrhagic disease was caused by an associated virus and not by sarcoma agent, Duran-Reynals made serial inoculations of extracts of viscera which showed the hemorrhagic lesions. This was done both in the young chicks and in the chick embryos. After 8 such serial transfers in the young chicks and 6 in the chick embryos, extracts of viscera with the hemorrhagic lesions repeatedly produced typical sarcoma when injected intradermally into adult chickens. Extracts of normal organs did not (4, 5). We have confirmed this in chick embryos following 10 serial transfers. The advantages in using this source of virus are twofold. First, it can be harvested under more sterile conditions. Secondly, it contains a relatively large quantity of active virus.

d. Purified virus suspension (CT #581): This was kindly furnished us as a standard for comparison by Dr. W. R. Bryan (9, 10, 11). It had been prepared from a citrate solu- of a tumor homogenate by repeated differential centrifugation. It was kept in 0.15 ml. amounts in sealed glass vials at dry ice temperature (−78°C).

2. Chick embryos: Fertile eggs of White Leghorn chickens were purchased from one commercial source. They were incubated at 37°–38°C and turned every two hours in an automatic Jamesway incubator before inoculation.

3. Method of extraction: A known weight of the tumor or chick embryo liver with hemorrhagic lesions was pulverized with sterile alundum sand in a mortar immersed in an ice-water bath. A measured amount of chilled buffered saline (0.066 M phosphate buffer, pH 7.2) was then added to make up a suspension of the desired concentration. This suspension was allowed to stand in the ice box (4°C) for 2 hours. It was then spun in an angle-head centrifuge at 2,000 r.p.m. for 15 minutes in the cold room (4°C). The supernatant was drawn off and used as the virus suspension. Further dilutions if desired were made up with chilled buffered saline. Aseptic technic was employed throughout these procedures and at all times the virus material was kept at 4°C.

4. Method of inoculation:

a. Chorio-allantoic membrane route of inoculation: The embryo was candled. A window was opened over an area showing the greatest vascularity, usually directly over the developing embryo. The chorio-allantoic membrane was dropped according to the method of Goodpasture and Buddingh (12). After inoculation, the window was sealed with a piece of Scotch tape.

b. Intravenous route of inoculation: The embryo was candled. The largest vessel adherent to the membrane was selected. A rectangular window was created in the shell, leaving the shell membrane intact. Depending on the age of the embryo, the inoculation was delayed for 4–8 hours or longer in order to minimize fatal traumatic bleeding at the time of inoculation. The vessel to be injected was made visible through the shell membrane by the application of a drop of sterile mineral oil. The desired amount of the virus suspension was injected through a 27 gauge needle. The window was then sealed with a piece of Scotch tape. Except for the 4–8 hour period of delay to minimize bleeding the method is essentially that of Eichorn's modification (13) of Polk, Buddingh and Goodpasture's method.

5. Observation routine: The chick embryos after inoculation were incubated at 38–39°C, except when indicated otherwise, without the periodic two hour turning. They were followed every day by means of candling. Deaths before the 5th day of incubation were not counted. This arbitrary rule was followed because, with the amount of virus used, few if any of the embryos observed developed lesions before the 5th day after inoculation. We cannot exclude, however, the possibility that these deaths could be due to the virus even

in the absence of grossly visible lesions. Ordinarily, most embryos inoculated by the chorio-allantoic membrane route survived, whereas, with the intravenous route of inoculation, 0–30 per cent of the embryos died during the first 4 days after inoculation. All embryos surviving past the 5th day following inoculation were carefully dissected when they died or were sacrificed at 19 days of embryo age, regardless of the age at which they were inoculated. A search for chorio-allantoic membrane tumor or hemorrhagic lesions was then made.

6. Amount of virus employed: In order to better demonstrate variables in host susceptibility, "Minimal infectious amounts" of the virus, i.e., amounts which produced milder lesions, were used. A desirable amount of the virus appeared to be one that produced lesions in 30 to 50 per cent of the embryos in the control group. Since extracts of different virus containing materials were used from one experiment to another, the dilutions of the extracts at which this desired effect was obtained varied. It would have been preferable to use a standard preparation such as the purified virus suspension.

RESULTS

1. Route of Inoculation

Since the focal point of study was hemorrhagic disease, intravenous injection seemed the route most likely to produce consistent lesions. This route proved so efficient in comparison with membrane inoculation that no other routes were explored. We found that although concentrated in the liver (Fig. 1), they were not limited to the embryo proper. Hemorrhagic blebs and spots appeared also along the blood vessels of the chorioallantoic and/or yolk sac. (Fig. 2).

2. Effect of Age of Embryo

The effect of the age of the embryos in producing lesions following membrane inoculation is most striking in the younger embryos according to our present limited data. Of 48 three day embryos, none developed membrane tumors and 23 developed hemorrhagic lesions in the embryo proper.* Six to ten day embryos showed a high percentage of membrane tumors, highest at day ten. The largest tumors were also seen at day ten. Roughly parallel percentages of hemorrhagic lesions were found within the embryos. By the twelfth day, the incidence of tumors and lesions dropped sharply. (Table II).

Study of the effect of embryo age in intravenous inoculation was somewhat limited by the technical difficulty of injecting the vessels of embryos of less than eight days. In a series of intravenously inoculated embryos aged eight to twelve days, the younger embryos yielded a higher percentage of lesions than the older. (Table III).

3. Effect of Temperature of Incubation

Chapman and Eaton found (8) that a temperature of 39°C is preferable for the development of lesions. Our results have simply confirmed their findings.

*Not all of these appear in Table II.

FIG. 1. Hemorrhagic lesions in the liver of chick embryo inoculated intravenously with Rous sarcoma virus—one hemorrhagic spot may be seen on apex of the heart.

FIG. 2. Hemorrhagic lesions in intact chorio-allantoic membrane of chick inoculated intravenously.

TABLE I

The Effect of the Route of Inoculation on the Development of Hemorrhagic Lesions

Experiment	Route of Inoculation	Total Number Embryos	Number of Embryos		No Lesions	Percentage with Lesions
			Hemorrhagic lesions	Chorio-allantoic membrane tumor		
Amount of virus: 0.05 ml. of 10^{-3} chick embryo liver extract. Age of embryos: 11 days old						
A	Intravenous	20	8	0	12	40.0
	Chorio-allantoic membrane	17	0	0	17	0.0
Amount of virus: 0.05 ml. of 10^{-3} dilution of CT # 581. Age of embryos: 10 days old						
B	Intravenous	9	9	0	0	100.0
	Chorio-allantoic membrane	18	2*	9	9	50.0

Temperature of incubation: 38° to 39°C.

* The hemorrhagic lesions appeared in embryos which developed chorio-allantoic membrane tumor.

TABLE II

The Effect of the Age of the Embryo on the Development of Lesions Following Chorio-allantoic Membrane Route of Inoculation

Experiment	Age of Embryos (days)	Total Number Embryos	Number of Embryos				Hemorrhagic Lesions	Percentage with Tumor	Percentage with Hemorrhagic Lesions
			Chorio-allantoic		Membrane	Tumor			
			0*	±	1+ to 2+	3+ to 4+			
A	3	5	5	0	0	0	2	0.0	40.0
	5	4	1	2	1	0	3	25.0	75.0
	7	5	0	2	3	0	5	60.0	100.0
	10	4	0	0	2	2	3	100.0	75.0
B	3	5	5	0	0	0	4	0.0	80.0
	4	5	4	0	1	0	4	20.0	80.0
	5	6	6	0	0	0	5	0.0	83.3
	6	4	2	0	2	0	2	50.0	50.0
	7	11	2	0	5	4	8	81.0	72.7
	8	9	1	0	6	2	8	88.8	88.8
	10	11	0	1	2	8	8	91.0	91.0
	12	7	3	2	2	0	2	28.6	28.6
	14	11	10	1	0	0	0	0.0	0.0
	16	10	10	0	0	0	0	0.0	0.0

Amount of virus: 0.05 ml. of 10^{-1} chicken tumor extract.

Temperature of incubation: 38°–39°C.

Route of inoculation: chorio-allantoic membrane.

* Size of chorio-allantoic membrane tumor is graded roughly on a 0–4 scale. 0 = no tumor. ± = thickening and edema of membrane at site of inoculation. 1+ to 2+ = definite tumor nodule to tumor less than 1 cm. in diameter. 3+ to 4+ = tumor 1 cm. and larger in diameter.

TABLE III

The Effect of the Age of the Embryo on the Development of the Hemorrhagic Disease Following Intravenous Route of Inoculation

Age of Embryos (days)	Experiment	Total Number Embryos	Embryos with Hemorrhagic Lesions	
			Number	Percentage
8	A	16	7	43.8
	B	19	9	47.4
9	A	11	1	9.0
	B	—	—	—
10	A	13	2	15.4
	B	30	3	10.0
11	A	16	1	6.2
	B	—	—	—
	A	17	0	0.0
	B	—	—	—

Amount of virus: in Experiment A: 0.05 ml. of 10^{-2} −4 chick embryo liver extract. In Experiment B: 0.05 ml of 10 dilution of CT # 581.
Temperature of incubation: 38° to 39° C.
Route of inoculation: intravenous.

TABLE IV

The Effect of the Temperature of Incubation on the Development of Chorio-allantoic Membrane Tumor

Temperature of Incubation	Total Number Embryos	Embryos with Chorio-allantoic Membrane Tumor	
		Number	Percentage
38–39°C	10	8	80.0
35°C	9	0	0.0

Amount of virus: 0.05 ml. of 10^{-1} dilution of chorio-allantoic membrane tumor extract.
Age of embryos: 10 days old.
Route of inoculation: chorio-allantoic membrane.

TABLE V

The Effect of the Temperature of Incubation on the Development of the Hemorrhagic Disease

Temperature of Incubation	Number of Embryos			Percentage with Lesions
	0*	1+ to 2+	3+ to 4+	
38–39°C	0	8	14	100.0
35°C	10	11	0	52.4

Amount of virus: 0.05 ml. of 10^{-3} dilution of CT # 581.
Age of embryos: 10 days old.
Route of inoculation: intravenous.
* Severity of hemorrhagic disease graded subjectively on a 0–4 scale.

We used 35°C as a comparative temperature because some viruses have produced better lesions at this degree of incubation (14).

4. Amount of Virus Inoculated

In general, a greater amount of virus produced a more severe infection. With minimal infectious amounts, embryos developed the hemorrhagic lesions but all survived a full nine day period of incubation after inoculation. With increasingly larger amount of virus inoculated, embryos died with more severe lesions and after increasingly shorter periods of incubation.

5. Sex of the Chick Embryo

No significant difference in the percentage of embryos showing the hemorrhagic lesions was found between embryos of the two sexes in our limited series, as indicated by the results presented in Table VI.

6. Treatment with Cortisone

Cortisone, at a dosage which produced marked stunting of embryo growth, reduced the percentage of embryos developing hemorrhagic lesions, as shown in Table VII. Embryos in the treated group were each given cortisone via the yolk sac at eight days of age while the controls were given an equal volume of saline. At ten days of age, both groups were inoculated intravenously with a virus suspension.

Most of the embryos which received either 0.6 mgm. or 1.3 mgm. of cortisone showed only slight to moderate cortisone effects: stunting of embryo growth, lack of feather formation, diminished yolk and albumen absorption and edema (15). Those that received 1.9 to 2.0 mgm. of cortisone showed marked cortisone effects.

7. Relation between the Severity of Hemorrhagic Lesions and the Amount of Virus Present

The amount of virus in the liver of embryos was measured at different intervals after inoculation. For these measurements we used the presence of

TABLE VI

The Effect of the Sex of Embryo on the Development of the Hemorrhagic Disease

Embryos with Lesions	Embryos without Lesions	Male	Female	Ratio M:F
21	—	13	8	1.6:1
—	37	19	23	1:1.2

Age of embryos: 11 days old.
Route of inoculation: intravenous.
Temperature of incubation: 38° to 39°C.

TABLE VII

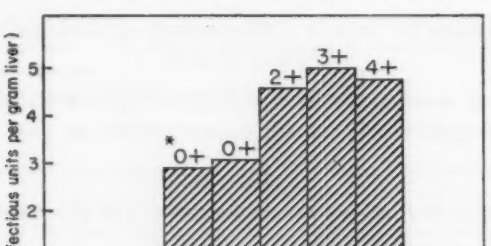
The Effect of Cortisone on the Development of the Hemorrhagic Disease

Experiment	Amount of Virus	Treatment of Embryos	Total Number Embryos	Number of Embryos Dead by 19th Day	Embryos with Lesions Dead and Sacrificed	
					Number	Percentage
A	0.05 ml of 10^{-3} dilution chick embryo	Cortisone 1.9 mgm.	6	5	2	30.0
		Cortisone 1.3 mgm.	8	7	7	88.9
		Cortisone 0.6 mgm.	10	7	6	60.0
		Saline	5	4	5	100.0
B	0.05 ml. of 10^{-3} dilution chick embryo liver extract	Cortisone 1.9–2.0 mgm.	27	25	3	11.0
		Saline	17	7	7	41.0
C	0.05 ml. of $10^{-3.5}$ dilution of CT #581	Cortisone 2.0 mgm.	22	18	9	41.0
		Saline	20	9	14	70.0

Route of inoculation: intravenous.
Temperature of incubation: 38° to 39°C.

hemorrhagic lesions as an end point (16). Twenty-one ten day old embryos were inoculated intravenously with a suspension of virus, and on given days after the inoculation, one embryo was sacrificed at random from the group. The severity of the lesions in the liver was noted and the amount of virus determined by titration. Embryos began to die of the infection on the sixth day after inoculation, but only living embryos were selected for titration. The histogram in Figure 3 correlates the period of infection and the degree of severity of the hemorrhagic lesions in the liver with the amount of virus in the liver.

Though many more embryos are needed to develop an accurate curve show-



* Severity of lesions in liver indicated by 0 – 4 +.

FIG. 3. Relation of the amount of virus recovered from the liver of 10 day old chicks inoculated intravenously with Rous sarcoma virus.

ing the increase in virus during the course of infection, a few conclusions may be drawn from the data at hand. Virus may be present in an appreciable amount in a liver without any grossly visible lesions. There is a latent period of probably several days following inoculation before grossly visible lesions appear. There seems to be a direct relationship between the amount of virus present and the duration of the infection as well as between the amount of virus and the severity of the lesions in the liver. In one instance, the virus content of a 4 plus hemorrhagic liver from a dead embryo was determined. The amount was lower than that of a liver showing the same severity of hemorrhagic lesions but from a live embryo. Rapid deterioration of the virus after cell death may account for the loss of titer.

DISCUSSION

The hemorrhagic lesions in chick embryos inoculated with Rous sarcoma virus are probably caused by the destruction of endothelial cells and subsequent extravasation, as suggested by Duran-Reynals (4, 5). Intravenously inoculated virus has direct access to these susceptible endothelial cells. The vessel selected for injection is usually a tributary of the umbilical vein. Virus is presumably carried by venous circulation back to the chick embryo. In a ten day old embryo, the umbilical vein empties into the ductus venosus which leads to the embryo heart. Most of the blood that reaches the heart enters directly into the arterial circulation without passing through the pulmonary system (17). The hemorrhagic lesions on the skin, in the body wall, on the spleen, liver, gizzard and intestines and along the vessels of the extra-embryonic circulation (both chorio-allantoic and yolk sac circulations) are probably started by virus particles which get into the arterial circulation and are caught in the peripheral capillary beds. These lesions may also serve as secondary sources of the virus. The exact cause of death of the embryo is not established. It may be due to the generalized virus infection and not, as has been suggested, (18) to acute blood loss following large scale extravasation from the hemorrhagic lesions. Many of our embryos that died with severe hemorrhagic lesions did not have free blood in either the abdominal or thoracic cavities.

There is a rather long latent period before grossly visible hemorrhagic lesions appear in the embryo after infection with the Rous sarcoma virus even when a relatively large amount of the virus is used. When the virus is inoculated subcutaneously into chickens there is also a latent period before lesions appear. There is again a latent period before definite degenerative changes appear in chicken fibroblasts grown in vitro and infected with the virus (20). These periods suggest either that the virus multiplies rather slowly in the susceptible cells or that the host cell tolerates relatively large amounts of virus without showing any morphological abnormalities.

We have shown that elevated temperature of incubation and use of younger

embryos are factors favoring the development of hemorrhagic lesions while treatment with cortisone is not. Do these factors act by altering cellular response to the virus or by altering the ability of the host cell to support virus multiplication? It is known from tissue culture work that the aviruleut strain of Newcastle disease virus destroys chick macrophages at higher temperatures of incubation but not at lower (21). The question of host cell response could be better answered if the amount of virus present had been determined (9). Probably both mechanisms play a part.

It should be noted that all three factors (temperature of incubation, age of the embryo and cortisone treatment) affect the general cellular metabolism. Whether the common basis for the specific effect of each factor is the general cellular metabolism will require further exploration. Tissue culture may prove an effective medium for differential study.

Andrewes (22) indicated that the yolk of eggs from immune hens contained a substance that neutralized Rous sarcoma virus, and other antibodies are known to occur in the yolk (23).

SUMMARY

The appearance of hemorrhagic lesions in the chick embryo infected with Rous sarcoma virus was favored by (1) an elevated temperature of incubation, (2) the intravenous route of inoculation, and (3) younger embryos. These lesions could be produced in a majority of cases when a sufficient amount of virus was injected intravenously into eight to ten day old chick embryos, incubated at 38° to 39°C. Sex of the embryo was not found to influence the development of lesions in our limited series. Cortisone at a dose that produced marked stunting of embryo growth reduced the percentage of embryos that developed hemorrhagic lesions.

Following intravenous inoculation of the virus, the hemorrhagic disease was not limited to the endothelial cells of the chick embryo proper but sometimes involved the endothelial cells of the extraembryonic circulation. The larger the amount of virus inoculated, the more severe was the infection.

Whether or not a chorio-allanotic membrane tumor occurs after inoculation of the Rous sarcoma virus onto the membrane depends on the age of the embryo. The best chorio-allantoic membrane tumors were seen in embryos inoculated on about their tenth day of age. In our series, none of the embryos inoculated on their third day of age showed any tumor formation. After twelve days of age, few showed any lesion.

BIBLIOGRAPHY

1. Rous, P. and Murphy, J. B.: Variations in a chicken sarcoma caused by a filterable agent. J. Exp. Med. 1913, **17:** 219.
2. Pentimalli, F.: Über metastasenbildung beim huhnersarkom. Z. Krebsforsch. 1925, **22:** 62.

3. SUGIURA, K.: The availability of young chicks for tumor transplantation. J. Cancer Res. 1926, **10,** 481.
4. DURAN-REYNALS, F.: A hemorrhagic disease occurring in chicks inoculated with the Rous sarcoma and Fuginami viruses. Yale J. Biol. and Med. 1940–41, **13:** 77.
5. MILFORD, J. AND DURAN-REYNALS, F.: Growth of a chicken sarcoma virus in the chick embryo in absence of neoplasia. Cancer Res. 1943, **3:** 578.
6. KARNOFSKY, D., RIDGWAY, L., AND PATTERSON, P.: Tumor transplantation to the chick embryo. Ann. New York Acad. Sc. 1952, **55:** 313.
7. OKER-BLOM, N.: Behavior of virus from chicken and duck tumors in embryonated egg of chickens and ducks. Acta path. microb. Scand., 1951, **28:** 1.
8. CHAPMAN, S. S., EATON, M. D. AND COLOMBO, F.: Personal communication.
9. BRYAN, W. R., MAVER, M. E., MOLONEY, J. B., THOMAS, M. A. AND SELLS, M. T.: Comparative stability of the agent of chicken tumor 1 in phosphate and borate buffers during incubation for 3 hours at 37°C. J. Nat. Cancer Inst. 1949, **10:** 647.
10. BRYAN, W. R., MAVER, M. E., MOLONEY, J. B., CALNAN, D., WHITE, C. L. AND WOOD, M. T.: Biological activity of the agent of chicken tumor (1) (Rous) in citrate buffers of various molar concentrations. J. Nat. Cancer Inst. 1951, **11:** 929.
11. BRYAN, W. R.: Personal communication.
12. GOODPASTURE, E. W. AND BUDDINGH, G. J.: The cultivation of vaccine and other viruses in the chorio-allantoic membrane of chick embryos. Science, 1931, **74:** 371.
13. EICHORN, E. A.: Technic for intravenous inoculation of chick embryos. Science, 1940, **92:** 245.
14. BEVERIDGE, W. I. B. AND BURNET, F. M.: *The Cultivation of Viruses and Rickettsiae in the Chick Embryo.* Spec. Rep. Series Med. Res. Council, No. 256, 1946, His Majesty's Stationery Office, London, England.
15. KARNOFSKY, D., RIDGWAY, L. AND PATTERSON, P.: Growth inhibiting effect of cortisone acetate on the chick embryo. Endocrinology, 1951, **48:** 696.
16. LO, WILSON H. Y. AND BANG, FREDERIK B.: Rous sarcoma virus infection in the chick embryo. II. A method of measuring virus activity and neutralizing antibody. Bull. Johns Hopkins Hosp. 1955, **97:** 238.
17. *Lillie's Development of the Chick*, 3rd edition. Revised by Howard L. Hamilton, 1952, Henry Holt & Co., New York, N. Y.
18. DURAN-REYNALS, F.: The significance of the non-neoplastic lesions induced in the central nervous system of ducklings by the virus of a duck variant of the Rous sarcoma. Yale J. Biol. and Med. 1950, **22:** 555.
19. BRYAN, W. R.: Quantitative studies on the latent period of tumors induced with subcutaneous injections of the agent of chicken tumor 1. 1. Curve relating dosage of agent and chicken response. J. Nat. Cancer Inst. 1946, **6:** 225.
20. LO, WILSON, H. Y., GEY, GEORGE O. AND SHAPRAS, PETER: The cytopathogenic effect of the Rous sarcoma virus on chicken fibroblasts in tissue culture. Bull. Johns Hopkins Hosp. 1955, **97:** 248.
21. BANG, F. B. AND WARWICK, A.: Fed. Proc. 1954, **13:** 1421.
22. ANDREWES, C. H.: The occurrence of neutralizing antibodies for Rous sarcoma virus in the sera of young "normal" chicks. J. Path. and Bact. 1939, **48:** 225.
23. BRANDLY, C. A., MOSES, H. E. AND JUNGHERR, E. L.: Transmission of antiviral activity via the egg and the role of congenital passive immunity to Newcastle disease in chickens. Am. J. Vet. Res. 1946, **7:** 333.

ROUS SARCOMA VIRUS INFECTION IN THE CHICK EMBRYO

II. A Method of Measuring Virus Activity and Neutralizing Antibody

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INTRODUCTION

The preceding paper described some conditions under which hemorrhagic lesions developed in chick embryos infected with the virus of Rous Sarcoma. Embryos infected with this virus develop lesions that are clear-cut and characteristic. The lesions do not occur spontaneously. Milford and Duran-Reynals found that only embryos having these definitive lesions yielded virus (2). Development of the lesions, then, might be a well-defined end point for titration of the virus.

It was found in this study that virus activity could be measured by inoculating ten day embryos intravenously with dilutions of the virus and incubating at 39°C. Neutralizing antibody was determined by testing dilutions of sera for their ability to neutralize a given quantity of virus.

MATERIALS AND METHODS

1. Source of Rous Sarcoma virus: Dr. W. R. Bryan of the National Cancer Institute provided the partially purified virus suspensions used in the titration and neutralization experiments reported in this paper. Three batches CT 522, 559, 581 were furnished in sealed glass vials, each of which contained 0.15 ml. of virus suspension. They were kept in a CO_2 box, and no loss of activity was detected. CT 559 and CT 581 contained an equivalent of 1 gm. chicken tumor material per ml. of virus suspension while CT 522 contained 2.5 gm. per ml. The nitrogen concentration, respectively, of CT 522, 559 and 581 are 0.05, 0.25 and 0.19 mgm. per ml. (5).

2. Preparation of virus dilutions for titration: The contents of each vial (0.15 ml.) were mixed with 14.85 ml. of chilled buffered saline to give a 1:100 dilution by weight. Further serial 10-fold dilutions were prepared with chilled buffered saline. These virus dilutions were kept in ice-water bath at all times.

3. Preparation of chicken sera for neutralization experiments: Chickens were bled from the heart after 24 hours' fasting. Blood was kept in the refrigerator @ 4°C for 24 hours and the serum then separated by centrifugation.

4. Preparation of serum-virus mixtures in neutralization experiments: A 1:50 dilution of the virus was made by mixing 0.15 ml. of CT 581 with 7.35 ml. of 10 per cent human placental cord serum. Human placental cord serum was found to contain no neutralizing antibody against the Rous sarcoma virus and was used to stabilize the virus. Equal amounts of the 1:50 virus dilution and the dilution of chicken serum were mixed and kept at 4°C for 4

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hours (6) before inoculation into chick embryos. The final virus concentration in the serum-virus mixtures was 1:100 of CT 581 in all the neutralization experiments. This was about 60 ID_{50}. A control preparation in 10 per cent human placental cord serum alone, was run in every experiment.

5. Chick embryos: Eggs from the same White Leghorn chickens were used throughout. Eight days old embryos were more susceptible to the virus, but many died during the first days after inoculation; and since hemorrhages were not present the virus could not be designated as the cause of death. For this reason ten day old embryos have been used.

6. Method of inoculation:

a. *Titration:* Using the same syringe and needle for the various dilutions, the most dilute preparation was inoculated first, and then the successively more concentrated. After thoroughly rinsing the syringe and needle with the new dilution 0.05 ml. of the dilution was inoculated intravenously into each embryo.

b. *Neutralization:* A different syringe and different needle were used for each serum-virus mixture. Otherwise, the method of inoculation was the same as in titration experiments.

7. Temperature of incubation of the chick embryos: 38–39°C (4).

8. Observation routine: This was described in the previous paper (2).

9. Method of calculation: The infectious dose 50 or ID_{50} was calculated by the Reed-Muench method (5). The standard error of the ID_{50} was calculated by means of the Pizzi (6) formula. The protective dose 50 or PD_{50} of each serum was calculated also by the Reed-Muench method.

RESULTS

1. Titration of Purified Virus Suspensions: The reproducibility of the titrations for three different virus preparations is presented in Table I. The mean ID_{50} of the three preparations studied were 4.4, 3.8 and 3.3. The mean interquartile range, i.e. the difference between the 25 per cent and the 75 per cent end point, R, was calculated to be approximately 1 log dilution of virus, and we have used this mean in our calculations. The interval between successive log dilutions of the virus, D, was also 1 log dilution. Hence by Pizzi's formula,

$$\text{S. E.}_{ID_{50}} = \frac{0.79 \times D \times R}{N} = \frac{0.79 \times 1 \times 1}{N}$$

where $\text{S.E.}_{ID_{50}}$ is the standard error of the ID_{50}, and N is the average number of chick embryos for each log virus dilution. When N is 5, the S.E. is about 0.4; when N is 10, and S.E. is about 0.3.

The actual range of variation in the three titrations was 0.6, which in most cases was carried out on less than ten embryos for each dilution and is thus less than two times the standard error. We conclude that the method of titration is reproducible within the limits imposed by the number of animals used. The percentage of embryos infected by each dilution of the standard preparation 581 is accumulated for the different titrations of this sample in Figure 1. The decrease in infectiousness on dilution, i.e., the titration curve, may be constructed from the three titrations as follows: the percentage of embryos that

TABLE I

Titration of Purified Virus Suspensions

Date	Log Virus Dilution	Number Embryos		ID_{50}	*R*	*N*	S.E.	Mean ID_{50}
		Infected	Non-infected					
1-26-54	2	4	0	3.5	0.7	6	0.3	3.8
	3	5	1					
	4	1	6					
	5	0	7					
	6	0	7					
4-8-54	2	6	1	3.5	0.9	7	0.3	
	3	5	1					
	4	2	6					
6-17-54	2	6	1	3.7	1.1	8	0.3	
	3	8	0					
	4	3	6					
	5	1	7					
6-20-54	2	9	1	4.1	1.2	10	0.3	
	3	9	1					
	4	6	4					
	5	1	9					
6-21-54	2	5	3	3.9	1.3	8.5	0.35	
	3	9	0					
	4	5	4					
	5	1	7					
6-24-54	2	11	0	4.1	1.2	10	0.3	
	3	9	0					
	4	5	5					
	5	2	9					
CT #559								
1-30-54	2	4	0	4.2	1.0	4.8	0.40	4.3
	3	3	1					
	4	4	1					
	5	0	6					
6-20-54	2	8	1	4.5	1.1	8.8	0.31	
	3	6	1					
	4	8	2					
	5	3	6					
6-21-54	2	8	1	4.0	1.5	9	0.36	
	3	7	2					
	4	5	4					
	5	2	7					
6-24-54	2	10	1	4.7	0.9	10	0.27	
	3	11	0					
	4	11	0					
	5	3	5					

TABLE I (*Cont'd.*)

CT #522

Date	Log Virus Dilution	Number Embryos		ID_{50}	*R*	*N*	S.E.	Mean ID_{50}
		Infected	Non-Infected					
2-4-54	2	4	1	3.2	1.	6	0.37	3.3
	3	5	2					
	4	0	7					
	5	0	5					
6-17-54	2	6	2	2.9	1.3	7.8	0.36	
	3	4	4					
	4	1	7					
	5	0	7					
6-20-54	2	8	1	3.5	0.6	9	0.23	
	3	10	0					
	4	1	8					
	5	0	8					
6-24-54	2	10	1	3.5	0.7	10.5	0.23	
	3	9	1					
	4	1	10					
	5	1	9					

R = interquartile range. *N* = average number per embryos dilution. S.E. = standard error of the ID_{50}.

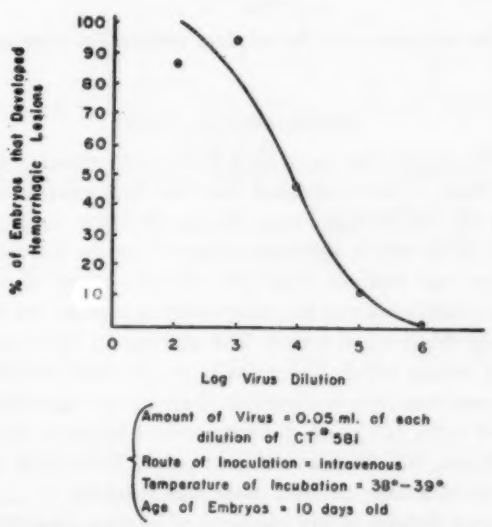

FIG. 1. Dilution end-point curve for standard preparation.

developed hemorrhagic lesions was plotted against a corrected value for the log dilution of virus. This correction was made by arbitrarily giving all three preparations the same 50 per cent end point and placing the other points correspondingly up or down.

It is seen that the curve closely approximates the theoretical Poisson curve based on the assumption that the infection is initiated by a single infectious unit. However, the more concentrated preparations of virus did not produce 100 per cent lesions. This may be related to individual variation in resistance.

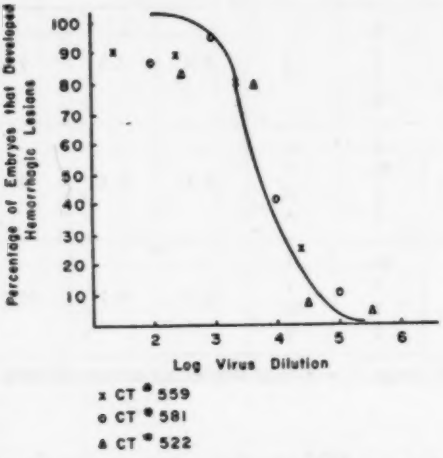

FIG. 2. Dilution end-point curve for all three preparations corrected to one arbitrary 50% end-point.

NEUTRALIZATION TESTS

Since the chick embryo has been used as the test animal in a number of viral neutralization tests, it was expected that the hemorrhagic lesions would be neutralized by chicken immune sera. For preliminary tests of neutralization, the serum of a chick which had been resistant to the intramuscular inoculation of the virus was studied. Since the effective range of dilutions of virus which could be inoculated into an embryo was not great, we have studied the effect of varying dilutions of serum. The amount of virus used was 60 ID_{50}. The dilution of serum which theoretically would have reduced the infection ratio to 50 per cent was then determined. It should be noted again that the dose of unneutralized virus, (60 ID_{50}), did not produce lesions in 100 per cent of the inoculated embryos, but 50 per cent end points have been determined here only in cases where a clear-cut end point was reached.

1. A preliminary survey of five chickens of various ages (Table II) showed that neutralizing activity was present in an eight month old "resistant"

TABLE II

Preliminary Survey for Neutralizing Antibody Against Rous Sarcoma Virus in Chickens of Various Ages (White Leghorns)

Age of Chickens	Serum Dilution	Number Embryos		PD_{50} Serum
		Infected	Non-infected	
8 months (resistant) 5-6-54	1:200	1	8	72.3
	1:20	0	8	
	1:2	0	6	
	Control	6	0	
5-22-54	1:20,000	9	0	2.5
	1:2,000	8	1	
	1:200	4	5	
	Control	4	1	
4 months	1:200	9	0	<0.3
	1:20	9	1	
	1:2	8	1	
	Control	6	2	
1 month	1:200	7	1	<0.3
	1:20	8	1	
	1:2	9	0	
	Control	2	0	
2 weeks	1:200	7	2	<0.3
	1:20	6	2	
	1:2	9	1	
	Control	—	—	
2 days	1:200	7	1	1.3
	1:20	3	4	
	1:2	2	6	
	Control	5	0	

TABLE III

Heat Stability of the Neutralizing Antibody in Serum of a Resistant Chicken*

Treatment of Serum	Serum Dilution	Number Embryos		PD_{50} Serum
		Infected	Non-infected	
Unheated serum	1:20,000	9	0	2.5
	1:2,000	9	1	
	1:200	4	5	
56°C for 30 minutes	1:20,000	7	0	2.8
	1:2,000	7	2	
	1:200	2	6	
75°C for 30 minutes	1:20	7	1	<1.3
Control	—	4	1	—

* This serum was obtained from the 8 month old resistant white Leghorn chicken, listed in Table II.

chicken and in a two day old baby chick. The latter may be explained by passive transfer of antibody through the yolk (8). To test the specificity of this neutralization, the heat lability of the presumed antibody was studied. Antibody may often be differentiated from non-specific neutralizing activity by the higher temperature at which it is inactivated. Heating at 56°C for 30 minutes did not inactivate the neutralizing activity (Table III) of this sera (9, 10) but the activity was destroyed by heating at 75° C for 30 minutes. This is true of other neutralizing antibodies (11).

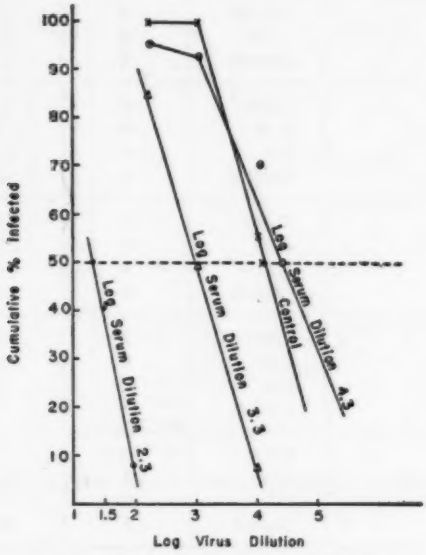

FIG. 3. Amount of virus neutralized by different serum dilutions.

The relation between the amount of virus neutralized and the dilution of serum used was studied within a range of three log dilutions of virus. The cumulative percentage of embryos infected at different dilutions of virus with three dilutions of serum was determined. The 50 per cent end point at a serum dilution of log 2.3 was log 1.2. At a serum dilution of 4.3 the 50 per cent end point was 4.3. Thus an increase of serum concentration of 100 fold (2 log) was accompanied by an increase in virus neutralized of 3.1 (or 1000 fold). The slope would then be 3/2 or 1.5. This is similar to other virus neutralization systems in the embryo. (Fig. 3).

Neutralizing antibody in tumor-bearing chickens: Sera drawn 3 to 4 weeks after inoculation of the virus into chickens were shown to neutralize the virus at various concentrations, as shown in Table IV. Sera drawn 10 days after virus inoculation contained no detectable antibody, even though the virus had pro-

TABLE IV

Neutralizing Antibody in Sera of Tumor-bearing Chickens

Chicken Number	Time of Collection	Serum Dilution	Number Embryos		PD_{50} Serum
			Infected	Non-infected	
1	3 weeks after virus	1:200	7	2	
		1:20	10	0	<0.3
		1:2	7	2	
2	After tumor regressed	1:200	8	1	
		1:20	4	5	1.5
		1:2	0	10	
		Control	9	1	—
	3 weeks after virus	1:200	7	1	
		1:20	3	3	1.8
		1:2	3	4	
	3–4 weeks after virus	1:200	5	4	
		1:20	2	8	2.0
		1:2	0	7	
		Control	10	0	—
5	10 days after virus	1:20	10	0	
		1:2	10	1	<0.3†
6	10 days after virus	1:200	7	3	
		1:20	10	1	<0.3†
		1:2	10	1	
		Control	12	0	—

* The same result was obtained when the serum was heated at 56°C for 30 minutes.

† Sera collected from these two chickens before virus inoculation also showed no protective antibody.

duced tumors in the chickens. The development of neutralizing antibodies in Rous sarcoma virus infection thus seems to be rather slow.

DISCUSSION

There is a considerable difference in susceptibility to the Rous sarcoma virus among chick embryos, even among those of the same batch. This had been observed also in baby chicks and adult chickens (10, 12). In spite of the lack of 100 per cent susceptibility, this present method of titration yielded highly reproducible results. The difference in the ID_{50}'s for each virus preparation was entirely within the range of experimental error.

In the conventional method of titration, whether using the serial dilution method of Carr (13) or the latent period method of Bryan (12), the test animal

is the young chick, and the end point the appearance of tumor at the site of inoculation. According to Bryan (5), two serious difficulties exist: first, there is a great deal of variability in the susceptibility of chickens, and the difference in chicken response becomes more apparent at higher dilutions. Second, regression of the tumor occurs frequently at higher dilutions of the virus, so it is impossible to tell whether the nodule is of inflammatory or neoplastic origin. The end point, therefore, is not clear cut.

It should be pointed out, however, that the sensitivity of our method of titration is considerably lower than that obtained by the conventional method. Whereas we obtained a value of about 5×10^5 infectious units per gram of tumor, the same preparation (CT 559) in the hands of Bryan (5), gave approximately 10^7 infectious units per gram of tumor. This indicates that an infectious unit for the endothelial cells of the chick embryo is considerably greater than one for the fibroblasts of the young chick. However, a tenfold difference in titer between CT 559 and CT 522 was detected by our method and this difference was confirmed by Bryan's method (5). The method of titration in our study seems therefore well suited for the determination of relative potencies of different virus preparations, and the saving in time and animal care are an added advantage.

It is of interest to note that the order of potency of the three virus preparations is in accord with the amount of N present. CT 559 has the highest N-concentration and is the most potent of the three whereas CT 522 has the lowest N-concentration and is the least potent. The loss in potency following repeated purification is well known (15).

The neutralizing substance in chicken serum behaved as an antibody; (a) it was present in a resistant chicken and a baby chick, which had probably acquired it via the yolk; (b) it was not altered by heating at 56°C for 30 minutes but was destroyed by heating at 75°C for 30 minutes, (c) a straight line relationship existed between the amount of virus neutralized and the serum dilution, and (d) it was present in tumor-bearing chickens (though not in the early stages of the infection). It has been reported present in the globulin fraction of the serum proteins (15). Whether there is a cross immunity between the Rous sarcoma virus and other fowl tumor virus infections (16, 17) is worth exploring with this new method of neutralization.

SUMMARY

A method of titrating the Rous sarcoma virus and measuring neutralizing antibody against this virus has been described. The development of hemorrhagic lesions following inoculation of the virus is used as the end point. Replicate titration of three purified virus preparations with this method gave results consistent within the expected chance variation.

Application of the method to the measurement of neutralizing antibody in chicken sera revealed that (a) neutralizing antibody was not present in all

chickens but was detected in a resistant chicken and a baby chick, (b) it was present in tumor-bearing chickens 3 to 4 weeks following virus inoculation, (c) it was not altered by heating at 56°C for 30 minutes but was destroyed to a large extent by heating at 75°C for 30 minutes and (d) the relationship between the amount of virus neutralized and the serum dilution approximated a straight line.

BIBLIOGRAPHY

1. Milford, J. and Duran-Reynals, F.: Growth of a chicken sarcoma virus in the chick embryo in the absence of neoplasia. Cancer Res. 1943, **3:** 578.
2. Lo, Wilson H. Y. and Bang, Frederik B.: Rous sarcoma virus infection in the chick embryo. I. Pathogenesis of the Rous sarcoma virus infection in the chick embryo with particular emphasis on the hemorrhagic disease. Bull. Johns Hopkins Hosp., 1955, **97:** 227.
3. Bryan, W. R., Maver, M. E., Moloney, J. B., Calnan, D., White, C. L. and Wood, M. T.: Biological activity of the agent of chicken tumor 1 (Rous) in citrate buffers of various molar concentrations. J. Nat. Cancer Inst. 1951, **11:** 929.
4. Bryan, W. R., Maver, M. E., Moloney, J. B., Thomas, M. A. and Sells, M.: Comparative stability of the agent of chicken tumor 1 in phosphate and borate buffers during incubation for 3 hours at 37°C. J. Nat. Cancer Inst. 1949, **10:** 647.
5. Reed, J. H. and Muench, H. J.: The simple method of estimating fifty per cent end points. Am. J. Hyg. 1938, **27:** 493.
6. Pizzi, M.: Sampling variation of the fifty per cent end-point, determined by the Reed-Muench (Behrens) method. Human Biol. 1950, **22:** 151.
7. Duran-Reynals, F.: Neutralization of tumor viruses by the blood of normal fowls of different ages. Yale, J. Biol. and Med. 1940, **13:** 61.
8. Andrewes, C. H.: The occurrence of neutralizing antibodies for Rous sarcoma virus in the sera of young "normal chicks." J. Path. and Bact. 1939, **48:** 225.
9. Des Ligneris, M. J. A.: Immunity in Rous sarcoma and its bearing on the problem of the nature of normal and cancerous growths. Publications of S. African Inst. Med. Res. 1934, **6:** 1.
10. Kenzy, S. G.: Studies in avian neoplasia. 1. A quantitative evaluation of neutralizing antibodies for the Rous sarcoma in avian serums. Am. J. Vet. Res. 1952, **13:** 388.
11. Kabat, E. A. and Mayer, M. M.: *Experimental Immunochemistry.* 1st edition, 1948, Charles C Thomas, Springfield, Illinois.
12. Bryan, W. R.: Quantitative studies on the latent period of tumors induced with subcutaneous injections of the agent of chicken tumor 1. 1. Curve relating dosage of agent and chicken response. J. Nat. Cancer Inst. 1946, **6:** 225.
13. Carr, J. G.: Relation between age, structure and agent content of Rous number 1 sarcoma. Brit. J. Exp. Path. 1943, **24:** 133.
14. Bryan, W. R., Riley, V. T., Deihl, D. G. and Voorhees, V.: Studies on the agent of chicken tumor 1. 1. The relative potencies of serial extracts and their comparative values as source materials for purification studies. J. Nat. Cancer Inst. 1947, **7:** 447.
15. Duran-Reynals, F.: A hemorrhagic disease occurring in chicks inoculated with the Rous and Fuginami viruses. Yale J. Biol. and Med. 1940–41, **13:** 77.
16. Andrewes, C. H.: The immunological relationships of fowl tumors with different histological structure. J. Path. and Bact. 1931, **34:** 91.
17. Foulds, L.: Observation on non-filtrable fowl tumor. Production of neutralizing sera against filtrates of Rous sarcoma 1 by non-infective extracts of a sarcoma induced by 1:2, 5:6 dibenzanthracene. Am. J. Cancer, 1937, **31:** 404.

THE CYTOPATHOGENIC EFFECT OF THE ROUS SARCOMA VIRUS ON CHICKEN FIBRCBLASTS IN TISSUE CULTURES

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INTRODUCTION

Since the discovery of the Rous sarcoma virus, efforts have been made to transform susceptible chicken cells cultured in vitro into sarcoma cells by exposing the tissue cultured cells to the agent. The results obtained by a number of workers are still conflicting. Basing his arguments on the ability of the "infected" cells or of the supernatant fluid from tissue cultures to produce tumors upon injection into chickens, Carrel (1, 2) claimed that macrophages supported the growth of the virus over a much longer period of time than did fibroblasts and hence should be considered the malignant cells of the Rous sarcoma, whereas, Ludford (3), and Sanford, Bryan, Earle et al. (4) claim that the fibroblasts supported the growth of the virus longer. Carrel (5) also claimed that the macrophages were transformed into fibroblasts under the influence of the Rous virus. However, he did not describe any cytological abnormalities in these infected cells. Halberstaedter, Doljanski and Tenenbaum (6) exposed fibroblast cultures to pieces of intensely irradiated Rous sarcoma cultures which presumably contained no living cells except the active virus and claimed that they observed cytological and cultural changes similar to those present in cultures of "pure" Rous sarcoma cells. Their work, however, is open to question on several points. First, they did not convincingly show that living cells were not present in the irradiated Rous sarcoma cultures which they used as the source of virus. It should be noted that the lethal dose of radium irradiation for normal chick embryo heart fibroblasts in tissue culture was found by Goldfeder (7) to be of the order of 10,000 mgm.-hr, a dose much greater than that used by Halberstaedter et al. in their experiment to kill the cells in Rous sarcoma cultures. Secondly, their cultures were often on the verge of death, requiring the addition of fresh tissues from time to time. In such degenerating cultures, degenerative cellular changes could be due to factors other than the virus. Thirdly, they did not present adequate photographic records to support their claims. On the other hand, in a later communication, Doljanski, Hoffman and Tenenbaum (8) reported no cytological or cultural changes in

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chicken fibroblast cultures grown in media containing Rous sarcoma extract. However, it is important to note that no tests were carried out to determine the virus activity of the extract.

We have been able to cultivate chicken fibroblasts in heterologous media over a prolonged period of time in a healthy condition. Some of these cultures were exposed to the Rous agent and definite cytopathogenic effects have been observed in such exposed cultures. These cytological changes were identical with those seen in cultures of the Rous sarcoma grown in vitro under the same conditions.

MATERIALS AND METHODS

1. Cultivation of chicken fibroblasts infected with the Rous sarcoma virus: Two separate attempts were made to infect chicken fibroblasts, using different virus preparations. The methods and materials used will be described separately.

Experiment A

(a) Chicken fibroblast cultures: Fragments of muscle from the anterior aspect of the right thigh of a three to four weeks old White Leghorn chicken were explanted into roller tubes. Abundant growth of fibroblasts was obtained and the fibroblastic outgrowth was serially subcultured in order to maintain the cells in a healthy condition. Repeated subcultures were necessary almost every four days during the four months of continuous cultivation. The cells remained in a healthy condition with the treatment and media to be described below. After the fourth month, degenerative changes set in and the cultures were then discontinued.

(b) Treatment of cells: The fibroblasts were grown in a clot substrate. The clot was obtained by mixing two drops of chicken plasma (CP) with three drops of a mixture containing one part of Gey's balanced salt solution (BSS), one part of beef embryo extract (BEE) and four parts of human placental cord serum (CS) (9). The final composition of the clot was therefore BSS 1: BEE 1: CS 4: CP 4. The supernatant nutrient fluid was composed of eight drops of a mixture containing BSS 5: BEE 1: CS 4 and two drops of a 0.02 per cent phenol red solution. The phenol red solution was made up by mixing 0.4 ml. of a 0.5 per cent phenol red solution (obtained from the Microbiological Associates Inc.) with 9.6 ml. of Gey's BSS. The final concentration of the phenol red in the nutrient fluid was 0.004 per cent. The fluid was renewed three times a week. Between renewals of fluid, the pH was adjusted by the addition of a 1.4 per cent $NaHCO_3$ solution (isotonic) (10). Ordinarily, one drop of $NaHCO_3$ was needed to bring the pH back to about eight when it had dropped to a value of around 7.2. Repeated subcultures were necessary almost every four days. About four to twelve hours before each treatment (renewal of fluid or subculture), the nutrient fluid was removed and Gey's BSS in equal volume added. Such four to twelve hour BSS washings helped to maintain the cells in a healthy condition, relatively free of droplets in the cytoplasm. The cultures were incubated at 37°C. The conditions of the cultures and the cells were observed daily and recorded.

Several attempts were made to maintain fibroblast cultures in entirely homologous media, using chicken plasma, chick embryo extract, chicken serum, all prepared from materials collected locally, and Gey's BSS. However, these cultures were always less healthy than those in heterologous media. In one comparative series, all cultures in homologous media died from some unknown cause whereas cultures in heterologous media remained

healthy and grew vigorously. The death of the cells in this series might have been due to an agent contributed by one of the homologous components, which, in the presence of heterologous components, was inhibited, as postulated by Gey and Bang (11). To avoid such difficulties, we have used heterologous media in combination with chicken plasma.

(c) Virus preparations: Rous sarcoma tissues were obtained from White Leghorn chickens of approximately five weeks of age. They were homogenized in a Sorvall blendor with nine parts of BSS for five minutes at 10,000 r.p.m. This 1:10 tumor extract was then passed through a medium sized Mandler bacteriological filter (bubbling pressure of 9 lbs.) which was previously demonstrated to be impervious to fresh culture of Serratia marcescens. The filtrate, containing active virus, was used for infecting chicken fibroblast cultures.

(d) Method of demonstrating Rous virus activity: The presence of active virus, either in the tumor filtrate or in the supernatant nutrient fluid of the infected cultures, was demonstrated by inoculating 0.05 ml. of the fluid intravenously into nine to eleven day old chick embryos. These inoculated embryos were incubated at 38° to 39°C (12). When active virus was present, a hemorrhagic disease of the chick embryo would become evident in five to nine days following inoculation, usually resulting in the death of the chick embryos. This method of demonstrating virus activity is elaborated in detail in a separate paper (13). Supernatant nutrient fluid withdrawn at different intervals from non-infected control cultures did not produce hemorrhagic lesions in the chick embryos whereas fluids from infected cultures did. In one instance, the supernatant nutrient fluids were inoculated subcutaneously into the anterior wing area of four to five week old White Leghorn chickens. Of the four cultures tested, fluid from two infected cultures produced tumors and fluids from two non-infected control cultures did not, within a period of examination of 30 days.

(e) Method of infecting the chicken fibroblast cultures: The cultures to be infected were usually about two days old following subculture. The supernatant nutrient fluid was withdrawn and an equal volume of the tumor filtrate was introduced. The cells were exposed to the filtrate for 24 to 48 hours. The controls were treated with a filtrate of a 1:10 BSS extract of normal chicken muscle. The infected cultures and their controls were maintained by the same method of cultivation as described for the chicken fibroblast cultures in (b).

Experiment B

(a) Chicken fibroblast cultures: These were obtained by explanting fragments of thigh muscle from a two day old chick into roller tubes.

(b) Virus preparation: "Purified virus suspensions" were kindly provided us by Dr. W. Ray Bryan of the National Cancer Institute. They were prepared from tumor homogenate by repeated differential centrifugation at high speeds after treatment with citrate solution. They are entirely free of living cells (14). They are distributed in 0.15 ml. amounts in sealed glass vials and stored at dry ice temperature (−78°C). The cultures were infected by introducing 0.15 ml. of the virus suspension into the supernatant nutrient fluid. The cells were exposed to this virus preparation for 24 hours.

Otherwise, the methods of cultivation and demonstration of virus activity were entirely similar to those used in Experiment A.

2. Cultivation of Rous sarcoma tissue: Primary explants were made of a huge Rous sarcoma in a ten to eleven week old White Leghorn chicken, which occupied all ot the left breast area. The method of cultivation was exactly the same as that for cultures of chicken fibroblasts described above, except that from time to time it was necessary to patch the clot in order to obtain an appreciable amount of outgrowth since the Rous sarcoma cultures lysed clots rapidly. Tests for Rous virus activity in the supernatant nutrient fluid were also made by inoculating chick embryos intravenously.

3. Method of cultivating cells on slide for phase microscopy: 2 by 3 inch stainless steel slides, two-thirds mm. thick, with a 35 mm. hole in the center were used. Two coverslips (40 by 45 mm.) were sealed on opposite sides of the slide, covering the central hole. The cells were grown between the two coverslips and in clot of the same composition as that used for roller tube cultures. The clot was covered by a layer of nutrient fluid (BSS 5: BEE 1: CS 4) which made contact with both coverslips, and the cells were therefore maintained in a partially anaerobic environment. The cells were observed under the phase microscope one to two days after transfer from roller tubes onto the slides. Details of this method have been described in previous communications (9, 15).

RESULTS

Experiment A

1. Protocol: Chicken fibroblast cultures were started on Jan. 1, 1954 and maintained in a healthy condition until April 26, 1954 when degenerative cellular changes set in. The cultures were terminated on May 11, 1954. One culture was successfully infected with tumor filtrate on Jan. 15, 1954 as shown by the fact that the supernatant nutrient fluid on Jan. 20, 1954 from a subculture made 2 days after exposure to the tumor filtrate was positive for virus activity. Since then, between Jan. and April, 1954, the supernatant nutrient fluids from serial subcultures were positive for virus activity on six separate occasions whereas fluids from the non-infected control cultures were negative for virus activity on each occasion. The last test for virus activity in the supernatant nutrient fluid was made 83 days after exposure to the tumor filtrate. The cells in these infected cultures grew vigorously and required subculturing almost every four days as did the non-infected control chicken fibroblast cultures. Another fibroblast culture of this series was successfully infected with tumor filtrate on Feb. 10, 1954. The supernatant nutrient fluid of its subcultures was last tested on May 2, 1954 and was positive for virus activity. The cells in this culture and its subcultures also grew vigorously. Early in May, 1954, these infected cultures began to grow poorly and soon could not be continued. Repeated loosening of the plasma clot on subculturing with rolling up of the fragments undoubtedly contributed to their demise.

2. Cytological and cultural characteristics of the non-infected control chicken fibroblast cultures: Attempts have been made to grow chicken fibroblasts on glass and in fluid media, both homologous and heterologous. However, the outgrowth was always much less abundant than cultures in plasma clot, especially when heterologous media were used. One difficulty encountered in such fluid cultures were that the continuity of the cellular outgrowth tended to break, leading to contraction and rolling up of the sheet of cellular outgrowth into a compact mass with death of many of the cells. Usually such cultures could not be maintained longer than four or five subcultures. In chicken plasma clot substrate, on the other hand, the chicken fibroblasts grew very well. The cells were quite uniform in shape, size and appearance (Fig. 1, Plate I). They

were spindle-shaped, quite slender aud their cytoplasm was almost devoid of droplets. If the clot was thin, lysis of the clot might occur where the outgrowth became abundant. In such lysed areas, degenerative cellular changes might appear, consisting mainly of an accumulation of droplets with slight distention and rounding up of the cell. If the clot was thick, lysis would not occur. In all our cultures, a *thick clot* was used. When the outgrowth became abundant, some cellular debris from degenerated cells could be found in all the cultures, but, in the main, the cells were healthy (Fig. 1, Plate IV and Fig. 1, Plate V) and many dividing cells were present. Such control non-infected cultures reduced the pH of the nutrient fluid very slowly. Only rarely did they require the addition of $NaHCO_3$ for pH adjustment.

3. Cytological and cultural changes in cultures infected with the Rous virus: Cytological alterations were first evident between ten to fourteen days after exposure to the virus. The earliest change consisted of the appearance of clumps of rounded up cells, apparently degenerating cells. These were found mostly in the intermediate zone of the outgrowth. Large amounts of cellular debris could also be seen (Fig. 2, Plate I). The central area of the colonies appeared much darker than those in the non-infected control cultures and apparently contained many necrotic cells. In contrast to the controls, lysis of clot occurred around almost every colony. After one or two more subcultures (Plate I, Figs. 2 to 4), hypertrophied bizarre looking cells appeared both in the area of clot lysis as well as at the periphery of the outgrowth. Around the periphery of the outgrowth, the gradual changes from a normal looking (Plate I, Fig. 3), slender, spindle-shaped fibroblast into the hypertrophied forms could actually be followed (Plate II, Figs. 1 to 4). However, it was usually in the area of clot lysis that the most severe degree of cellular hypertrophy was evident. The hypertrophy appeared to be the result of distention of the cell from the accumulation of large numbers of droplets in the cytoplasm. It was not unlike the degenerative cellular changes that one sometimes observes in the non-infected control cultures, except that it was of much greater severity. Under the phase microscope, the nuclei of these hypertrophied cells seemed to be pushed towards the periphery by the accumulated droplets. Most of these abnormal cells eventually disintegrated. With the passage of time, the severity of such changes became more marked. Some of the cells actually became converted into giant cells, without even the slightest suggestion of their original spindle-shaped form (Plate II, Figs. 3 and 4). One characteristic was always preserved; there was always a polymorphism of many cells in all the infected cultures. Normal appearing, slender, spindle-shaped cells as well as cells exhibiting varying degrees of alteration were to be found in the same culture On the other hand, in the non-infected control cultures, the cells were more or less uniform in shape, size and appearance as emphasized above. Under higher magnification, with living cells under the phase microscope (Exp. B, Plate IV,

Figs. 2 and 3, and Plate V, Figs. 2 to 4), many of the cells in the infected cultures were seen to be multinucleated. Some possessed five nuclei though binucleated cells were more frequent. These cytological changes were present in the infected cultures during the entire period of cultivation.

Another characteristic of the infected cultures was that the pH of the supernatant nutrient fluid dropped much more rapidly than that of the non-infected control cultures, requiring adjustment with $NaHCO_3$ frequently. Despite this rapid pH drop, the infected cultures showed no evidence of an increased proliferative capacity.

Experiment B

1. Protocol: Chicken fibroblast cultures were started on May 1, 1954. The cells were in a healthy condition throughout the entire period of the experiment. They were discontinued on June 17, 1954 when the experiment was terminated. Five of the cultures were exposed to Bryan's "purified virus suspension" on May 13, 1954 and all were successfully infected. These infected cells grew vigorously throughout the period of the experiment. The supernatant nutrient fluids from these infected cultures and their subcultures were found to be positive for virus activity when tested on three separate occasions during this period of maintenance, whereas fluids from the non-infected control cultures were negative. The last such test was carried out when these cultures were discontinued with their non-infected control cultures at the end of the experiment.

2. Cytological and cultural characteristics of the non-infected control chicken fibroblast cultures: These were identical with those described in Experiment A.

3. Cytological and cultural changes in the cultures infected with the Rous virus: The earliest response was seen four to five hours after exposure to the virus preparation as contrasted to the ten to fourteen day latent period described above in Experiment A. In several of the exposed cultures, many cells around the periphery of the outgrowth rounded up and were apparently dead. However many of the cells survived and proliferated at an unabated rate. Soon the dead cells disintegrated and were replaced by vigorously growing healthy cells from the expanding outgrowth. The earliest definite cellular changes contributed directly to the virus were noted eight days after exposure to the virus preparation. The cytological and cultural changes of these infected cultures were identical with those described in Experiment A. (See Plates IV and V.)

Characteristics of Rous sarcoma cultures

1. Protocol: Primary explants of Rous sarcoma were made on May 19, 1954. The cells grew vigorously. Because of the rapid lysis of clot, repeated patchings were necessary in order to maintain an appreciable outgrowth of cells. The cultures were terminated on June 14, 1954 at the end of the experiment. The supernatant nutrient fluid from serial subcultures were found to be posi-

tive for virus activity when tested on three separate occasions during the period of cultivation. The last test was carried out when the cultures were terminated.

2. Cytological and cultural characteristics: From the original explants some macrophages as well as the spindle-shaped cells grew out (Plate III, Fig. 1). Some hypertrophied cells (Plate III, Fig. 2) of a similar description as those mentioned under Experiments A and B appeared. However, after one or two subcultures, the macrophages disappeared from the cultures and the cultures were composed almost entirely of the spindle-shaped cells (Plate III, Figs. 2 and 3) and some abnormal looking cells of varying degrees of hypertrophy. The cultural and cytological characteristics of the cultures at this time were identical with those of the infected cultures in the early period of cultivation as described in Experiments A and B.

DISCUSSION

The controversy as to whether the fibroblast or the macrophage is the malignant cell in the Rous sarcoma has not been completely resolved. The ability of one or the other cell type to support the growth of the Rous virus for a long period of time has been the basis of this controversy. Tenenbaum and Doljanski (16) have described the morphology of cells in "pure" Rous sarcoma cultures. If one or the other cell type could be changed morphologically by the virus in vitro into altered cell forms such as seen in a Rous sarcoma culture, the question could then be answered decisively. However, it is quite possible that cell types other than the macrophage and fibroblast could be affected by the Rous sarcoma virus as well, e.g., the endothelial cells (17).

The morphology of the living cells in the Rous sarcoma cultures in our experiments fits closely the description given by Tenenbaum and Doljanski for living and stained cells. In all of these experiments the question may still be raised, "Is there a 'Rous sarcoma' cell?". The evidence based on tissue culture observations at present still points to its non-existence. There is always a heterogeneity of cell forms in a Rous sarcoma culture, varying from normal appearing fibroblasts to hypertrophied, multinucleated giant cells without the slightest suggestion of a spindle-shape form. This work shows that chicken fibroblast cultures could be morphologically changed into Rous-sarcoma-like cultures by exposure to the Rous virus. This, coupled with the work of Ludford (3), and of Sanford, Likely, Bryan and Earle (4) in which it was demonstrated that the fibroblasts supported the growth of the virus for a much longer period of time than the macrophages, seems to favor the argument that the fibroblast is the "malignant cell' or carrier of the virus in a Rous sarcoma. However, the final answer will have to await proof that other cell types could or could not be similarly transformed by infection with the Rous virus in vitro.

The rapid drop in pH of the supernatant nutrient fluids in the Rous virus infected cultures and Rous sarcoma cultures as compared to that of the non-

infected control fibroblast cultures is of considerable interest. These infected cultures as well as the Rous sarcoma cultures showed no evidence of increased proliferative capacity as compared to the non-infected control fibroblast cultures. Three possibilities exist to account for this phenomenon. First, that the Rous virus infected fibroblast is a truly malignant cell with a greater propensity for anaerobic metabolism, which yields large quantities of acid end-products (i.e. lactic acid). Second that the liberated Rous sarcoma virus and/or some component of the disintegrated infected cells are highly acidic. Third, that the lysis of plasma clot by certain enzymes released by the infected cells and the sarcoma cells results in some highly acidic substance or substances. Further work needs to be done to determine which of the three possibilities is true.

SUMMARY

Adult chicken fibroblast cultures have been maintained in a healthy condition in heterologous media for various periods of time. Some of these cultures have been successfully infected by both Rous sarcoma filtrate and Bryan's "purified virus suspension". The cells in the infected cultures showed the first evidence of a cytopathogenic effect of the virus at about eight to fourteen days following virus exposure. At first, degenerating cells appeared, leading to much cellular degeneration and necrosis. Yet serial subcultures could be made from these degenerating infected cultures and vigorously growing, apparently healthy cell colonies were obtained. Soon after transfer hypertrophied and even giant cells appeared, loaded with inclusion droplets. Later large numbers of these multinucleated giant cells were seen. Such severely altered cells eventually disintegrated. There was no evidence that any of these altered cells or the unaltered, apparently healthy cells had an increased proliferative capacity as compared to the non-infected cells. The morphological alterations in the infected chicken fibroblast cultures were identical with those seen in cultures of Rous sarcoma tissue. It is apparent from these observations that one of the important effects of the Rous sarcoma agent on chicken fibroblasts in tissue cultures is a severe cytopathologic change in the cells. Another important observation was the rapid drop in pH of the virus infected and the Rous sarcoma cultures.

BIBLIOGRAPHY

1. Carrel, A.: Action de l'extrait filtré du sarcome de Rous sur les macrophages du sang. Compt. rend. Soc. biol. 1924, **91,** 1069.
2. Carrel, A.: Effets de l'extrait de sarcomes fusocellulaires sur des cultures pures de fibroblastes. Compt. rend. Soc. biol. 1925, **92,** 477.
3. Ludford, R. J.: The production of tumors by cultures of normal cells treated with filtrates of filterable fowl tumors. Am. J. Cancer, 1937, **31,** 414.
4. Sanford, K. K., Likely, G. D., Bryan, W. R. and Earle, W. R.: The infection of cells in tissue culture with Rous sarcoma virus. J. Nat. Cancer Inst. 1952, **12,** 1317.

5. CARREL, A. AND EBELING, A. H.: The transformation of monocytes into fibroblasts through the action of Rous virus. J. Exp. Med. 1926, **43,** 461.
6. HALBERSTAEDTER, L., DOLJANSKI, L. AND TENENBAUM, E.: Experiments in the cancerization of cells in vitro by means of Rous sarcoma agent. Brit. J. Exp. Path. 1941, **22,** 179.
7. GOLDFEDER, A.: Studies on the effect of radiation upon growth and respiration of various tissues in vitro. The lethal dose and the sublethal dose of X-rays and radium: Preliminary report. Radiology, 1938, **31,** 73.
8. DOKJANSKI, L., HOFFMAN, R. S. AND TENENBAUM, E.: The effect of tumor extracts on the growth of cells in vitro. Growth, 1944, **8,** 13.
9. GEY, G. O. AND GEY, M. K.: Maintenance of human normal cells and tumor cells in continuous culture. Am. J. Cancer, 1936, **27,** 45.
10. GEY, G. O., HANKS, J. H. AND BARRET, R.: Retardation of growth and metabolism of normal and malignant cells during continuous cultivation. Growth, 1948, **12,** 69.
11. GEY, G. O. AND BANG, F. B.: Viruses and cells. A study in tissue culture application. 1. Cells involved—availability and susceptibility. Tr. New York Acad. Sc. 1951, **14,** 15.
12. CHAPMAN, S. S., EATON, M. D. AND COLOMBO, F.: Personal communication.
13. LO, W. H. Y. AND BANG, F. B.: To be published.
14. BRYAN, W. R.: Personal communication.
15. BANG, F. B. AND GEY, G. O.: Comparative susceptibility of cultured cell strains to the virus of Eastern equine encephalomyelitis. Bull. Johns Hopkins Hosp. 1952, **91,** 427.
16. DOLJANSKI, L. AND TENENBAUM, E.: Studies on Rous sarcoma cells cultivated in vitro. 2. Morphologic properties of Rous cells. Cancer Res. 1943, **3,** 585.
17. MILFORD, J. AND DURAN-REYNALS, F.: Growth of a chicken sarcoma virus in the chick embryo in absence of neoplasia. Cancer Res. 1943, **3,** 578.

PLATE I

FIG. 1. Subculture of control non-infected fibroblasts, 12 days after treatment with filtrate of normal muscle extract, showing the intermediate zone of outgrowth. Note the abundant outgrowth of healthy, spindle-shaped cells and the minimal amount of cellular debris. Roller tube culture. Magnification: See 200 micron scale.

FIG. 2. Subculture of Rous virus-infected fibroblasts, twelve days after virus exposure, showing the intermediate zone of outgrowth. Note clumps of rounded up, degenerating cells and the large amount of cellular debris. Roller tube culture. Same magnification as in Fig. 1.

FIG. 3. Subculture of Rous virus-infected fibroblasts, 20 days after virus exposure, showing a peripheral zone of outgrowth. Note the apparently normal appearing cells with no discernible morphological alterations due to the virus. Roller tube culture. Same magnification as in Fig. 1.

FIG. 4. Subculture of Rous virus-infected fibroblasts, fourteen days after virus exposure, showing an area of clot lysis. Note clumps of rounded up cells. Some of the cells here and in other areas show early hypertrophy. Roller tube culture. Same magnification as in Fig. 1.

FIG. 1

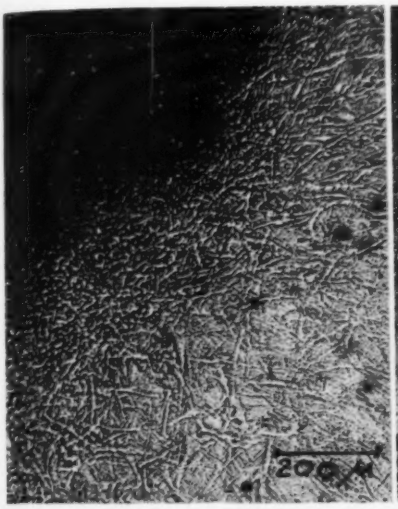


FIG. 2

FIG. 3

FIG. 4

PLATE II

FIG. 1. Subculture of Rous virus-infected fibroblasts, eighteen days after virus exposure showing an area of clot lysis. Note the polymorphism of cells, varying from normal appearing cells to greatly hypertrophied cells loaded with highly refractile droplets Note also scattered necrotic cells. Roller tube cultures. Magnification: See 200 micron scale.

FIG. 2. Subculture of Rous virus-infected fibroblasts, eighteen days after virus exposure, showing a peripheral zone of outgrowth. Note the transformation from normal appearing cells to hypertrophied forms with accumulations of highly refractile droplets. Roller tube culture. Same magnification as in Fig. 1.

FIG. 3. Subculture of Rous virus-infected fibroblasts, 45 days after virus exposure, showing an area of clot lysis. Note clumps of degenerating cells and round hypertrophied cells with highly refractile droplets. Roller tube culture. Same magnification as in Fig. 1.

FIG. 4. Subculture of Rous virus-infected fibroblasts, 79 days after virus exposure, showing an area of clot lysis. Note the large numbers of giant cells loaded with highly refractile droplets. Roller tube culture. Same magnification as in Fig. 1.

FIG. 1

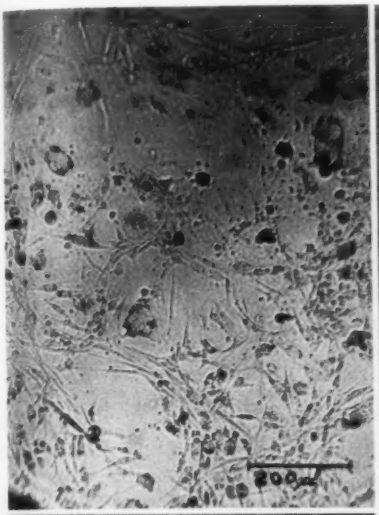


FIG. 2

FIG. 3

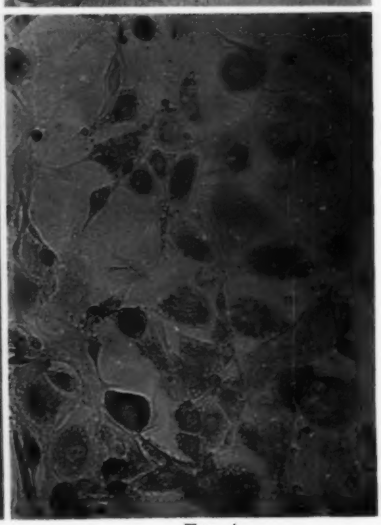


FIG. 4

PLATE III

FIG. 1. Subculture of Rous sarcoma cells, 12 days after primary explantation, showing an area of clot lysis. Note clumps of rounded up, degenerating cells and some apparently healthy cells. Roller tube culture. Magnification: See 200 micron scale.

FIG. 2. Subculture of Rous sarcoma cells, 18 days after primary explantation, showing an area of clot lysis. Note the polymorphism of cells, varying from normal appearing, spindle-shaped cells to greatly hypertrophied cells loaded with highly refractile droplets. Roller tube culture. Same magnification as in Fig. 1.

FIG. 3. Subculture of Rous sarcoma cells, 18 days after primary explantation, showing a peripheral zone of outgrowth. Note the apparently healthy "sarcomatous" fibroblasts in intact plasma clot. Roller tube culture. Same magnification as in Fig. 1.

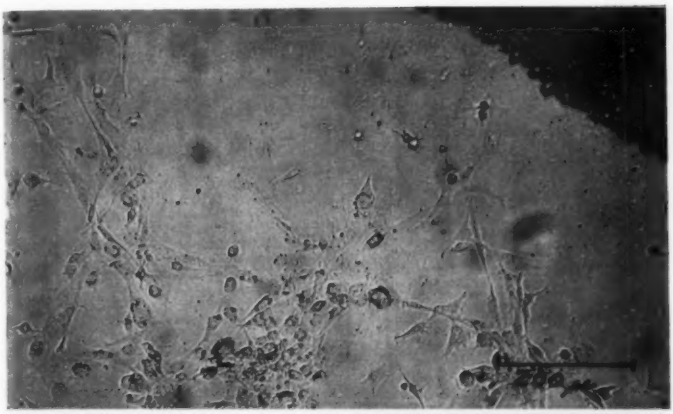


FIG. 1

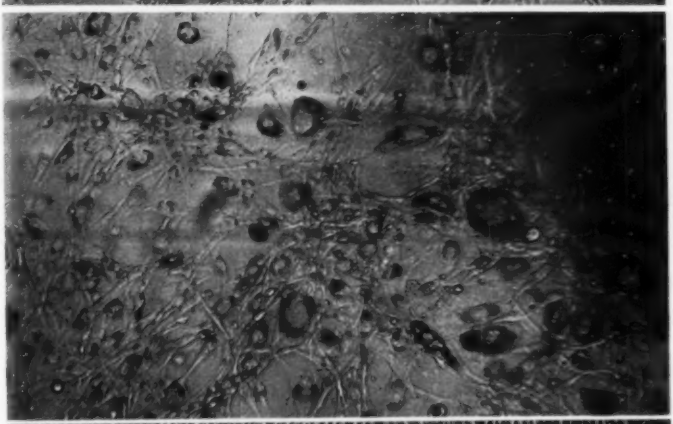


FIG. 2

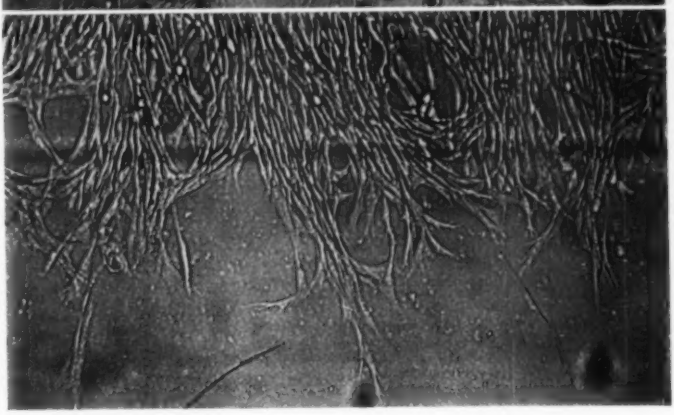


FIG. 3

PLATE IV

FIG. 1. Slide culture of control non-infected chicken fibroblasts, prepared 27 days after primary explantation. Note that the cells are healthy and fairly clear with few inclusion droplets. Two dividing cells are present. Magnification: See 50 micron scale. Phase image. 4 mm. Bright M objective (American Optical Co.).

FIG. 2. Slide culture of Rous virus-infected fibroblasts, prepared fourteen days after virus exposure. Note the cellular debris, degenerating cells and swollen vacuolated cells. Same magnification as in Fig. 1. Phase image. 4 mm. Bright M objective (American Optical Co.).

FIG. 3. Slide culture of Rous sarcoma cells, prepared ten days after primary explantation. Note the enormously hypertrophied binucleated cell, loaded with highly refractile droplets. Other smaller cells are present with fewer inclusion droplets. Phase image. 4 mm. Bright M objective (American Optical Co.).

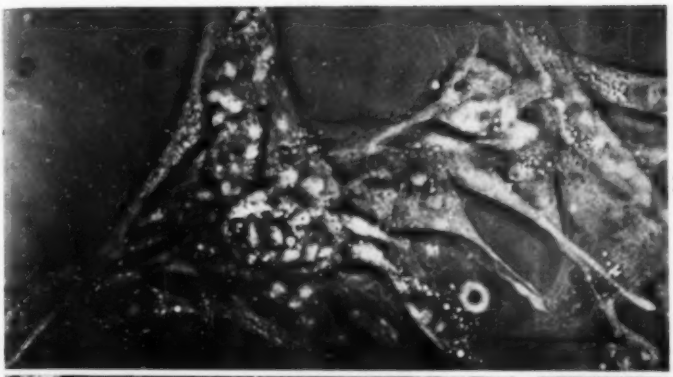


FIG. 1

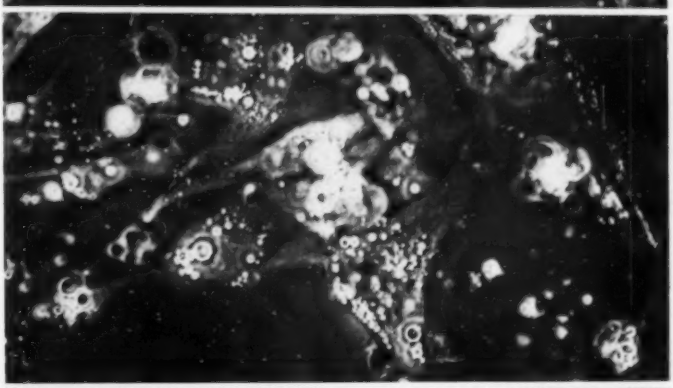


FIG. 2

FIG. 3

PLATE V

FIG. 1. Slide culture of control non-infected fibroblasts, prepared 28 days after primary explantation. Note the relatively clear ground cytoplasm, the numerous filamentous mitochondria, the small numbers of inclusion droplets and the sharp nuclear membranes of these two normal cells. One nucleus shows two compact rounded nucleoli whereas the nucleus of the other cell shows two expanded nucleoli of irregular shape actually made of a loose arrangement of nucleolar strands. Magnification: See 20 micron scale. Phase image. 1.8 mm. Bright M objective (American Optical Co.).

FIG. 2. Slide culture of Rous virus-infected fibroblasts, prepared fifteen days after virus exposure. Note the vacuolar changes in the cytoplasm. Many intact filamentous mitochondria were found to be present. Same magnification as in Fig. 1. Phase image. 1.8 mm. Bright M objective (American Optical Co.).

FIG. 3. Slide culture of Rous virus-infected fibroblasts, prepared fifteen days after virus exposure. Note the hypertrophied cell, distended with many inclusion droplets which surround the nucleus and the juxta-nuclear area (giant centrosphere). This cell also contains many intact filamentous mitochondria. Same magnification as in Fig. 1. Phase image. 1.8 mm. Bright M objective (American Optical Co.).

FIG. 4. Slide culture of Rous sarcoma cells, prepared 10 days after primary explantation. Note the greatly hypertrophied cell, showing changes similar to the cell in Fig. 3. This binucleated cell has many healthy filamentous mitochondria and numerous inclusion droplets. Phase image. 1.8 mm. Bright M objective (American Optical Co.).

FIG. 1

FIG. 2

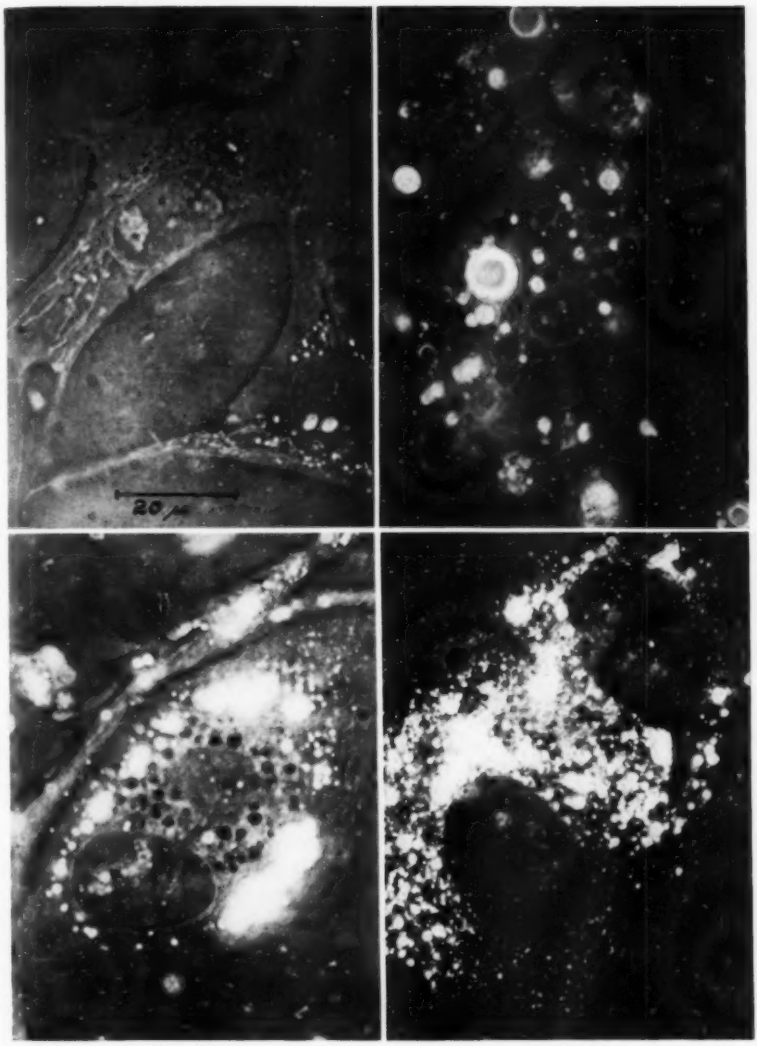
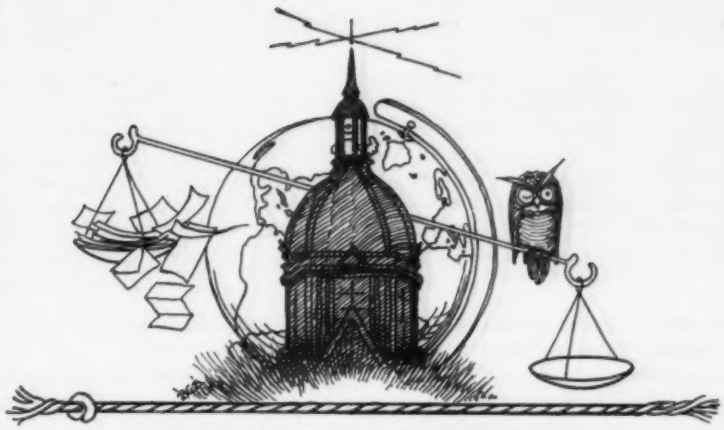


FIG. 3

FIG. 4



BOOK REVIEWS

Experiencing the Patient's Day. A Manual for Psychiatric Hospital Personnel. By ROBERT W. HYDE. 214 pp., $2.20. G. P. Putnam's Sons, New York, N. Y.

As fewer and fewer psychiatric hospitals are organized after the pattern of jails, hospital administrators have given serious thought and effort to the matter of orientation and instruction of psychiatric attendants. Although it is doubtless true that no sure-fire, universally satisfactory formula for accomplishing this has yet been worked out, this manual is worth noting as an attempt to point a way. The author, who is Assistant Superintendent of the Boston Psychopathic Hospital, has found it helpful if psychiatric aides and attendants gathered in informal weekly discussion groups with hospital psychiatrists and other experienced personnel. These discussions were focussed on the patient's life in the hospital, "his relationship to personnel and on exploring our own life as personnel within the mental hospital, what patients mean to us, our concerns about them and how we feel in response to their varied attitudes toward us." This book reports some of the results of this educational experiment.

In each chapter a brief résumé of the topic for discussion is followed by a verbatim account of the discussion that ensued. The chapters are short and readable and deal with topics ranging from the mundane problems of the patients' 24-hour day through matters related to examination, treatment, symptoms and diagnoses and special problems of ward behavior. Each of the 48 topics that came under discussion drew upon the experiences of and the problems encountered by individual attendants in their work with hospitalized patients. Such discussions thus had vastly greater pertinence and interest to the participants than any didactic lecture could have been expected to have. The author feels that "the most promising source of attendant education is that which comes from the attendant himself."

Although it is doubtless possible for attendants to work for years in a psychiatric institution without gaining a glimmering of understanding of their charges, it is the considered opinion of many responsible psychiatrists that it is the attendant or the nurse who has an interest in and understanding of patients who, in the end, contributes indispensably toward a patient's progress or recovery and the smooth-running of a psychiatric hospital. In some professionally understaffed hospitals this contribution is virtually all that the majority of chronic patients can hope for by way of individual attention. If discussion groups of the kind described in this book can keep alive a curiosity and a desire to understand among the attendant staff, not only will it enrich the experience of the psychiatric attendant but patients will profit as well.

This book, despite incompleteness in scope, is oriented toward helping attendants understand more of the psychodynamics of personality functioning. It provides many ideas that should prove extremely useful for those psychiatrists interested in the education of psychiatric attendants and other personnel. The accounts of group discussions reported in this book demonstrate with clarity what one hospital has been able to accomplish with the method. In the opinion of the reviewer this book should be used as a guide to educators, offering them a method to follow; it is not precisely a manual, as its title claims, for it would not be suitable for the education of attendants without an accompanying program of group discussions.

JOHN L. HAMPSON

Living Bone in Health and Disease. By IRVIN STEIN, RAYMOND O. STEIN AND MARTIN L. BELLER. 510 pp., $15.00. J. B. Lippincott Co., Philadelphia, Pa.

"Living Bone" is a textbook of medical diseases of bone. Although it is fundamentally a clinical textbook primary emphasis is placed on the histology, biochemistry and physiology of bone. The first section, of eighty-four pages, deals exclusively with the structure and physiology of normal bones while the next four hundred pages discuss specific pathological states which involve bone either primarily or secondarily. These include both rare and common diseases in sections generally appropriate in length to their clinical importance. A short final section is composed of tables and a system of differential diagnosis.

In writing this book the authors have transgressed the customary clinical divisions and gathered together in one volume information on bone usually divided among publications of endocrinologists, orthopaedists, biochemists, etc. In doing so they have produced a volume which will be valuable for all, though not definitive for any. It is written in good, clear, though occasionally abrupt English, and is well organized and up to date. The book is copiously illustrated; well printed and bound. It is recommended to clinicians who see diseases involving the skeletal system, as a very practical reference work, and as an excellent textbook of its subject.

GEORGE SETTLE

Fractures in Children. By WALTER P. BLOUNT. 279 pp., $9.50. The Williams & Wilkins Co., Baltimore, Md.

This volume is a fairly comprehensive treatise on fractures in children but differs from the usual textbook in the manner in which the material is presented. As one reads it, one gets the impression that the author is discussing with a class of students his experiences and observations in the handling of children's fractures. He glosses over the heavier aspects and emphasizes the more practical matters, particularly the differences between the problems in childhood and in adult life.

The writer succeeds in making the book quite readable by his relatively informal style, by the use of numerous good and well-reproduced illustrations, and by the introduction o. occasional funny cartoons that emphasize his "don'ts". One might question the need for a book devoted only to the fractures in children. The author anticipates this criticism and offers his reasons for this specialized effort. In the opinion of this reader, the volume serves a useful purpose.

I. WILLIAM NACHLAS

Fibrosis of the Liver in West African Children. Medical Research Council Special Report Series No. 285. By J. H. WALTERS AND J. C. WATERLOW. 71 pp., 8s. 6d. net. Her Majesty's Stationery Office, London, England.

In this monograph, Walters and Waterlow present the thesis that the fibrosis of the liver common in children in the Gambia are the result of the combined effects of malnutrition and

malarial infections. These conclusions are based primarily on histologic and epidemiologic evidence. The view is of considerable interest. Even in classic experimental dietetic cirrhosis, one is never sure whether the lesions observed might not be due to a concomitant infection. It is hoped that the thesis will be tested experimentally. Most important would be to feed experimental animals the native diets. There is evidence from several laboratories that a cirrhosis-like lesion may be produced in rats by feeding human diets similar to those ingested by patients with cirrhosis. These diets contain supposedly adequate amounts of choline, methionine and cysteine, and demonstrate that there may be many pathways to a nutritional cirrhosis other than those usually described.

Finally it is noteworthy that the case fatality rate for liver biopsy in this series was 1 of 79 infants. This accords with the Cassandra-like views of some of us that punch biopsy of the liver is not to be regarded as a benign procedure.

OSCAR D. RATNOFF

Surgical Nursing, 10th edition. By ELDRIDGE L. ELIASON, L. KRAEER FERGUSON AND LILLIAN A. SHOLTIS. 754 pp., $4.75. J. B. Lippincott Co., Philadelphia, Pa.

The tenth edition of this book is a good revision. It has been reset in more legible type which accounts mostly for the increase of twenty-six pages. The old chapter seven on methods of Surgical Nursing has been omitted and Nursing Care has been correlated to a greater degree with the surgical aspects in the specific chapters. There have been a number of new headings throughout the book. The clinical situations at the end of the chapters have been revised.

New chapters have been introduced as follows: Postoperative Discomforts and Complaints,—which is the old chapter on Postoperative Complications; Special Therapies, Fluid Balance and Gas Therapy, Nursing Care of the Geriatric Surgical Patient and Surgery of the Chest.

Sections which have been rewritten in a large measure are: Applications of Heat and Cold, Sterilization Dressings, The Nurse and the Cancer Patient. The Appendix on Sterilization is also new.

In addition, new discussions which have been introduced are: d-tubocurarine chloride, artificial hibernation, artificial hypotension during operation, responsibility of the anesthesia service, postoperative reception of the patient, body alignment and good body mechanics, diagnostic aids (in cardiovascular surgery), the nurse and the urologic patient, chalazia, rhizotomy and cordotomy, miscellaneous foot conditions, vaginitis and safety practices in the operating room.

A good effort has been made in emphasizing the psychological aspects in the general discussions of the preoperative and postoperative care, and in The Nurse and the Cancer Patient. In the discussions of the care of the patients with specific problems the emphasis seems to be more diffuse. There is need for amplification and clarification in order to define what these aspects are. Additional reading is necessary, especially in the care of the patient with colostomy, radical mastectomy and surgery of the heart and great vessels.

This book continues to be one of the leading texts in the field. This edition serves to bring it up-to-date.

DORIS DILLER

Fluid and Electrolytes in Practice. By HARRY STATLAND. 206 pp., $5.00. J. B. Lippincott Co., Philadelphia, Pa.

This is another of the growing number of books, monographs, and primers dealing with acid base and electrolyte disturbances. The present volume is intended for practicing physi-

cians rather than for medical students. It is indicated on the dust jacket that the complex subject described has been rendered "intelligible to all, no matter how far removed from their studies in the basic sciences". If this aim has been achieved it has been by a superficial and in many instances completely inadequate treatment of the fundamentals. In addition, too few examples of specific clinical problems are presented; most disorders are discussed in terms of generalities which may be misleading in a field where few patients present a textbook picture. Finally, there is quite inadequate emphasis of the hazards associated with the injudicious use of ammonium chloride in patients with kidney or liver disease, of mercurial diuretics in patients with kidney disease, and of hypertonic salt solutions in patients with low serum sodiums. This book cannot be recommended.

F. P. CHINARD

BOOKS RECEIVED FOR REVIEW

The Adopted Break Silence. The experiences and views of forty adults who were once adopted children. Compiled and written by JEAN M. PATON. 180 pp., $3.00. Life History Study Center, Philadelphia, Pa.

The Biologic Effects of Tobacco. The First Complete Analysis of a Vital Subject. Edited by ERNEST L. WYNDER. 215 pp., $4.50. Little, Brown & Co., Boston, Mass.

Doctor and Patient. By DESMOND O'NEILL. 197 pp., $5.00. J. B. Lippincott Co., Philadelphia, Pa.

The Halogenated Hydrocarbons Toxicity and Potential Dangers. By W. F. VON OETTINGEN. 430 pp., $2.50. Public Health Service Publication No. 414. United States Government Printing Office, Washington, D. C.

Laboratory Exercises in Anatomy and Physiology. By DOROTHY WALTON PARRY. 158 pp., $3.00. G. P. Putnam's Sons, New York, N. Y.

Lehrbuch der Praktischen Orthopädie. By WALTHER ABERLE, PHILIPP ERLACHER, ALEXANDER HARTWICH, ALFONS KÖNIGSWIESER AND OSKAR STRACKER. 549 pp., $24.50. Wilhelm Maudrich, Vienna, Austria. Intercontinental Medical Book Corp., New York, N. Y.

Teaching Physiology and Anatomy in Nursing. By HESSEL H. FLITTER AND HAROLD R. ROWE. With Special Reference to the Textbook Physiology and Anatomy, by Esther M. Greisheimer, 7th edition. 56 pp., $2.00. J. B. Lippincott Co., Philadelphia, Pa.

Viral Hepatitis. Clinical, Laboratory and Public Health Aspects. Public Health Service Publication No. 435. By HEINZ F. EICHENWALD. 59 pp., 55 cents. United States Government Printing Office, Washington, D. C.